THE EFFECT OF KINETIN AND SALT STRESS ON PNP-A EXPRESSION IN *Erucastrum strigosum* AND *Arabidopsis thaliana*

By

Gile Dineo Makgoke

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Supervisor: Prof. C. A. Gehring
Co-supervisor: Dr. S. Rafudeen

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PREFACE

"I declare that, THE EFFECT OF KINETIN AND SALT STRESS ON PNP-A EXPRESSION IN *Erucastrum strigosum* AND *Arabidopsis thaliana*, is my own work and that all the sources I have used or quoted have been indicated and acknowledged by means of complete references."

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ABSTRACT

In agriculture crop productivity is greatly affected by stresses such as salinity, drought, temperature and hormonal changes of crop plants and responses to these stresses. Studies have shown that a natriuretic peptide based regulatory system responsible for water and ion homeostasis in animals has a heterologous equivalent in plants. Plant natriuretic peptide immunoreactants (irPNPs) have been reported to be involved in K^+, Na^+ and Cl^- ions fluxes in plants. Previously, an *Arabidopsis thaliana* transcript (AtPNP-A) encoding an irPNP (AtPNP-A) has been identified and isolated (Ludidi *et al.*, 2002). The AtPNP-A a novel protein and part of its physiological role is presented here.

Following on from these studies we sought to characterise the effect of salt and kinetin stress on PNP-A expression in *A. thaliana* and *Erucastrum strigosum* both belonging to the same plant family (Brassicaceus). To determine the effect of salt and salt-kinetin treatment on *A. thaliana* plants, seeds were germinated on media treated with different salt concentrations (50, 100, 150, 200) mM NaCl with and without 10μM kinetin respectively and grown for 24 days. When comparing salt-treated and salt-kinetin treated plants it was found that plants grown on salt-kinetin treated media were more susceptible to salt than plants grown in only salt treated media. Plants treated only with salt were greatly affected by the 150 mM and 200 mM NaCl treatment, with the percentage growth decreased from 80% to 60% and 40% respectively. Plants treated only with kinetin weighed less than untreated plants and the growth percentage was 37.5 %. This indicated that the amount of kinetin added was contributing to the salt toxicity. The plants treated with salt-kinetin had the growth

percentage reduced from 70% at 50 mM NaCl + 10 µM kinetin to 20 % at 200 mM NaCl + 10 µM kinetin. The salt-kinetin treatment therefore increased the salt toxicity effect.

Analyses of the K content of untreated and treated *A. thaliana* plants using atomic absorption analyses revealed that untreated plants had more K than salt treated plants. The salt treated plants had in turn more K than salt-kinetin treated plants. The K content decreased in both the salt and salt-kinetin treated plants at 0, 50 and 100 mM NaCl concentrations but increased at 150 mM NaCl in both salt and salt-kinetin treated plants. This increase corresponded to the salt inhibition observed in the plant growth studies. Analyses of the Na content in the plant revealed that it was the least in untreated plants. The salt treated plants had less Na than their counterpart salt-kinetin treated plants at similar (50, 100 and 150) mM NaCl concentrations. There was also a gradual increase in Na content in both the salt and salt-kinetin treated plants at 50, 100 and 150 mM NaCl concentrations. The plants that had the highest concentration of Na were those treated with 200 mM NaCl. Plants treated with 200 mM salt-kinetin were the most susceptible to salt.

We isolated and characterized the *PNP-A* gene from *E. strigosum* using RT-PCR. We found that the *PNP-A* gene had 98.95 % identity with the *A. thaliana PNP-A* gene, was 381 bp in length, and encoded a predicted *E. strigosum* PNP-A protein (EsPNP-A) with 99.21 % identity to the AtPNP-A protein. The predicted EsPNP-A protein had a single conservative amino acid change at position 111 when compared to the AtPNP-A protein. The predicted molecular weight of the protein is 14 kDa and predicted length is 126 amino acids. Phylogenetic analyses also revealed that the EsPNP-A protein belonged to the irPNP protein

family. These results indicate that the PNP-A gene occurs on the *E. strigosum* genome and the near identical conservation of amino acids at the protein level indicated that the *E. strigosum* PNP-A protein shares a similar if not identical function as the *A. thaliana* PNP-A protein.

The EsPNP-A protein was successfully extracted from untreated and treated *E. strigosum* plant leaves grown for 35 days and western blot analyses revealed that PNP-A was present in the crude protein with molecular weight of approximately ~14 kDa. This result together with the sequence analysis confirmed that PNP-A is present in *E. strigosum* plant leaves.

In order to determine the regulation of the *E. strigosum* PNP-A protein expression, BIOCAD affinity purification and western blot analysis was done on protein extracted from untreated and treated *E. strigosum* plant leaves. It was found that immunoreactants in both salt and salt-kinetin treated plants was highest at 300 mM NaCl treatment indicating that it was up-regulated at 300 mM NaCl and could therefore be involved in ion homeostasis in the plant.

The EsPNP-A protein was further characterized using BIOCAD affinity purification, western blot and subsequent MALDI-TOF analyses. It was found that the EsPNP-A in untreated *E. strigosum* plants yielded two protein bands with molecular weights of ~ 13 kDa and ~10 kDa. The ~13 kDa protein band could represent the EsPNP-A protein with the signal peptide (13 998 Da) and the ~10 kDa protein band could represent the EsPNP-A without the signal peptide (3 949 Da) (Ludidi *et al.,* 2002). This indicates that PNP-As exist in two forms in untreated *E. strigosum* plants. Plants treated with salt-kinetin revealed the presence of two

protein bands having a molecular mass of 8926.2 and 9440.71 Da respectively. These proteins were eluted from the anti-ANP column and thus the two bands could be two differently processed forms of the EsPNP-A protein.

RNA slot blot analyses was done to see whether the up-regulation of the EsPNP-A protein at 300 mM salt stress was reflected at the transcriptional level using a AtPNP-A and actin RNA probe. RNA slot blot analysis revealed that there was a three-fold increase in specific RNA levels in 300 mM NaCl treated plants as compared to control.

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ABBREVIATIONS

µM	Micro molar
µl	Micro liter
mM	Millimolar
NaCl	Sodium Chloride
g	Gram
ml	milliliter
L	liter
SDS	sodium dodecyl sulphate
V	volts
mA	milliamps
TBS	Tris buffered saline
T	Tween 20
mg	Milli grams
KCl	Potassium Chloride
EDTA	Ethylene Diamine Tetra-Acetic Acid Disodium Salt
MS	Murashige and Skoog Basal
APS	Ammonium Persulfate
TEMED	N,N,N,N'- Tetra- methyl-ethylenediamine
°C	Celsius
nm	Nanometers
rpm	Revolutions per minute

Na	Sodium
K	Potassium
NBT	Nitroblue tetrazolium salt
BCIP	5-bromo-4-chloro-3-indolyl phosphate
TFA	Trifluoroacetic acid
Acrylamide/Bis	N,N'-Methylene-bis-acrylamide

1. Introduction

The development and the growth of plants are influenced by a number of environmental and intracellular factors such as salinity, drought and hormones respectively. A change in any of these factors may have a negative or positive impact on the physiological status of the plants. The plants must respond to these factors either by adaptation or tolerance to the changes in order to avoid plant death. A critical understanding of plant stresses is necessary since they are key components to successful plant breeding in order to select those traits that will enhance the plant competency and increase yields which will in turn preserve the plant natural resources.

1.1 Salinity

Salinity stress is one of the most serious factors limiting the productivity obtainable from agricultural crops. Increased salt concentrations in the soil environment cause water deficit resulting in osmotic stress effecting critical biochemical processes. The stress is primarily caused by high sodium ions (Na^+) and chloride ions (Cl^-) concentrations in the soil solutions which impact negatively on critical metabolic processes (Wyn, 1981).

Salt movement into roots and shoots results from the transpirational flux which is required to maintain water in the plant (Hasegawa *et al*., 1994; Yeo, 1998). Unregulated transpiration can result in accumulation of ions in the aerial part of the plant which is toxic to the plant. An immediate response to an increase in salinity to the shoot is the closure of the plant stomata in order to offset the severity of the ion influx. However, because of the water potential difference between the leaf cells and the atmosphere and

the need for carbon fixation, this is an untenable long-term strategy for salt tolerance (Munns *et al.*,1986 ; Yeo, 1998). In order to protect developing and metabolizing cells plants regulate ion movements into tissues (Munns, 1993). One mode of action that the plant uses to control the salt flux to the shoot is the entry of ions to the xylem stream. At the endodermis the radial movement of the solutes must be through a symplastic pathway as the casparian strip serves as a physical barrier to apoplastic transport (Clarkson *et al.*, 1991). The accumulation of ions in mature and old leaves which then dehisce have also been observed under salt stress (Munns, 1993). In their function as ions sinks, the leaves that are old may be limiting the deposition of the ions into the meristem, growing and photosynthesizing cells. An alternative possibility is that cellular ion discrimination is a natural consequence of transpirational and exposure growth fluxes, cell morphology and the degree of intercellular connection. Meristematic cells which are not directly connected to the vasculartur, are minimally exposed to ions delivered via the transpiration stream, and their small vacuolar space is not conducive to the sequestration and storage of ions. The solute content in tissues containing cells with little vacuolation is predominated by organic osmolytes and in tissues with highly vacuolated cells ions are the major osmolyte (Binzel *et al.*, 1988; Wyn, 1981).

1.2 Salt tolerance among different plant species

For optimal growth the halophytes require electrolyte (typically Na^+ and Cl^-) concentrations higher than those found in non saline soils. They appear to lack a unique metabolic machinery that is activated or deactivated by sodium ions (Na^+) and chloride ions (Cl^-) (Nelson *et al.*, 1998; Niu *et al.*,1995; Rhodes and Samaras, 1994). The

halophyte plants survive and grow in saline environments due to osmotic adjustment via intracellular compartmentation that partitions the ions that are toxic away from the cytoplasm via the energy-dependent transport into the vacuole. (Apse *et al.*, 1999; Binzel *et al.*, 1988; Glenn *et al.*, 1999; Hajibagheri *et al.*, 1987; Niu *et al.*, 1995; Storey *et al.*, 1983; Yeo, 1998). Some halophytes make use of glands and bladders to exclude the sodium ions (Na^+) and chloride ions (Cl^-). In both the halophytes and glycophytes the osmotic adjustment is obtained through solute accumulation in the cytosol, lumen, matrix or stroma of organelles (Niu *et al.*, 1995; Rhodes *et al.*, 1994 and Yeo *et al.*, 1998). The major difference between the halophytes and the glycophytes is the ability of halophytes to survive salt shock. This allows the halophytes to readily a establish metabolic steady state for growth in a saline environment (Braun *et al.*, 1986; Casas *et al.*, 1991; Hassidim *et al.*, 1990 and Niu *et al.*, 1993). In glycophytes, ion movement is restricted to the shoot in order to control the influx of ions into the xylem of the root. The halophytes however tend to readily take up sodium ions (Na^+) in a manner that the roots have a lesser concentration of NaCl than the rest of the plant. (Adams *et al.*, 1992). The advantage that the halophytes have over the glycophytes is not only that they are more capable of Na^+ partitioning but also have a more effective way to coordinate this partitioning with processes controlling growth, and ion flux across the plasma membrane in the plant (Adams *et al.*, 1992; Glenn *et al.*, 1999; Greenway and Munns, 1980; Yeo *et al.*, 1998).

1.3 Plant Hormones

Plant hormones are chemical messengers which are produced in a specific part of the plant which can control the activity in a distal part of the plant (Jacobsen *et al.*, 1995). Their target of action is plant growth and differentiation which includes; cell elongation, cell division, shoot or root formation and stomatal movement in response to environmental stimuli. Plant hormones are classified into three main groups the first being the "*classical*" hormones namely the auxins, gibberellins (GA), cytokinins and abscisic acid (ABA) (Westhoff *et al.*,1998). The second group is the "*non*" classical which includes the oligosaccharins (Ryan,1988; Hahn *et al.*,1989; Ebel and Cosio, 1994; Pressey, 1991), brasssinolides (Sakurai and Fujioka, 1993), jasmonates (Creelman *et al.*, 1992; Bewly 1997; Berger *et al.*, 1996; Falkenstein *et al.*, 1991; Creelman and Millet, 1997); salicylic acids (Hunt *et al.*,1996; Yalpani *et al.*, 1991) and polyamines (Kakkar and Rai, 1993). The last group contains regulating peptides and includes systemin (Pearce *et al.*, 1991), ENOD40 (van de Sande *et al.*, 1996; Fang and Hirsch, 1998), phytosulfokines (Matsubayashi *et al.*, 1997; Matsubayashi and Sakagami, 1996) and the plant natriuretic peptides (Vesley and Giordano, 1992; Vesley *et al.*, 1991, 1993; Billington *et al.*, 1997). Only the first and the last group will be discussed since they are of great importance to this study.

1.3.1 Gibberellins (GA)

The gibberellins (GAs) are a large group of compounds which share the presence of a gibbane ring structure and have all similar biological activities (Sponsel,1995). Hisamatsu *et al.*, 1998 recently characterized GAs identified in higher plants species and

found that only a few had GA activity while others were precursors or deactivated GAs. The activity of gibberellin biosynthesis was found to be high in immature seeds particularly in the endosperm (Sponsel, 1995). The use of mutants and inhibitors revealed that the biosynthesis of GAs in young tissues of the shoot and developing seeds can be divided into three different stages (Hedden and Kamiya, 1997; MacMillan 1997). In the first stage geranyl geranyl diphophate (GGP) is converted to ent-keurene and the reaction is catalysed by enzymes called ent-keurene synthase A and B. In the second and third stage the formation of GA_{12} – aldehyde results from the ent-keurene rings rearrangement which then leads to the formation of various gibberellins via a series of different pathways. Gibberellins (GA3) are involved in the promotion of shoot growth, induction of flower formation in rosette plants and breaking seed dormancy (Westhoff *et al.*, 1998).

1.3.2 Auxin

In plants, IAA occurs in a free form and also in a series of conjugated amino acids, peptides or carbohydrates. It is involved in plant growth stimulation by elongation, formation of adventitious and side roots, maintenance of apical dominance, inhibition of leaf abscission and parthenocarp (Westhoff *et al.*,1998).

Indole-3-acetic acid (IAA) is the major natural auxin and its synthesis involves multiple formation synthetic pathways including several tryptophan-independent and tryptophan-dependent pathways namely via indole-3-pyruvic acid, tryptamine or indole-3-acentonitrile (Bandurski *et al.*, 1995). For example in the *Arabidopsis* system four nitrilase encoding genes that are involved in the conversion of indole-3-acetonitrile to IAA have been cloned (Bartel and Fink, 1994). Following the IAA hormone the tryptophan-independent pathway may be accessible from a non-tryptophan

precursor, such as via indole-3-methyl glucosinolate found in *Arabidopsis thaliana* (Normanly *et al.*, 1993). In the orange pericarp mutant of maize it was also found that even though there was no tryptophan production, higher levels of IAA hormone was being accumulated than in the wild type (Wright *et al.,* 1991).

1.3.3 Cytokinins

Cytokinins are involved in the stimulation of the plant cell division, lateral and shoot induction and retardation of senescence (Westhoff *et al.*, 1998).

In plants, cytokinins are N6-substituted adenine derivatives. Kinetin although not identified in plants is the most biologically active cytokinin. The biosynthesis of cytokinins has been found to be present in shoots, developing embryos and root apices (Letham *et al.*, 1978) even though other plant organs have been identified to have the ability to convert radiolabelled cytokinins precursors to active ones (Chen *et al.*, 1985). Transportation of cytokinins in plants from the roots to the leaves takes place in the transpiration stream through the phloem pathways and reaches all the cells present in the plant by diffusion (Kamboj *et al.*, 1998). The source of the cytokinins detected in the phloem saps however remains unresolved.

1.3.4 Abscisic acid (ABA)

Abscisic acid is a single hormone of a 15-carbon acid which in structure is related to part of the carotene molecule (Zeevaart and Creelman, 1988). ABA biosynthesis is divided into two steps which involve the xanthoxin conversion to ABA-aldehyde followed by ABA formation from ABA-aldehyde (Schwartz *et al.*, 1997). The plant leaves and the root cap are the sites in which the ABA biosynthesis is most extensive. ABA is involved in the enhancement of fruit and leaf

abscission, inhibition of seed dormancy, water stress and stomatal closure (Westhoff *et a*l.,1998). Like ethylene, ABA is produced in response to environmental signals particularly to water stress when the turgor reduction results in massive ABA production in the plant leaves and roots

1.3.5 Ethylene

Ethylene is a classical hormone which occurs in a gaseous form. For its removal metabolism is not required because of being a gas. Ethylene readily diffuses between cells present in the plant and to nearby plants. The biosynthesis of ethylene is extensive in the apical buds, stem nodes, senescing and fruit ripening. Ethylene has a role in enhancing fruit ripening, fruit and leaf abscission (Westhoff *et al.*, 1998).

In most plant tissues subjected to stress, ethylene is derived from methionine through the intermediate precursor 1-aminocyclopropane-1-carboxylic acid (ACC) through a process involving several steps. In the first step 5-adebosyl-L-methionine is converted to 5'-methylthioadenosine and 1-aminocyclopropane-1-carboxylic (ACC) which is catalyzed by enzyme ACC synthase and is followed by the conversion of ACC which is catalyzed by ACC oxidase enzyme (Abeles *et al.*, 1992).

1.4 Effects of classical hormones on stomatal movements

All classical hormones are involved in the stomatal movements by affecting the opening and closing of the stomata. It has been found that at low concentrations IAA opens the plant stomata (Marten *et al.*, 1991) and also activates $I_{(k+in)}$ thereby providing a pathway for the K^+ influx (Blatt and Thiel, 1994; Grabov and Blatt, 1997). However exogenous

application of auxin at high concentrations, from 100μM and above was found to promote stomatal closure (Marten *et al.*, 1991) and the inhibition of $I_{(k+in)}$ allowing K^+ efflux (Blatt and Thiel, 1994).

With ABA stomatal closure is promoted when the plant is exposed to environmental stresses such as high salinity and drought. These environmental conditions also tends to increase the synthesis of ABA with a resulting increase in distribution and accumulation (Sibole *et al.*,1998). The action of ABA in stomatal closure has also been found to be related to the increase of the cytoplasm pH between 0.2 and 0.4 units (Irving *et al.*, 1990; Blatt and Thiel,1993); $I_{(k+out)}$ activation (Blatt and Amstrong,1993) inward K^+ channels inhibition (Blatt,1990); plasma membrane proton pump inhibition reduced the proton motive force for K^+ and Cl^- uptake (Gohl *et al.*,1996;) supported this finding by showing inhibition of the plasma membrane proton pump by ABA reduces a proton motive force for K^+ and Cl^-.

Stomatal opening has been found to be promoted in the presence of both naturally occurring cytokinins and synthetic ones in the epidermal peels e.g. (Blackman and Davies, 1983; Marten *et al.*,1991; Pharmawati *et al.*, 1998a).

1.5 Aspects of the signal transduction pathways

1.5.1 Auxin

Exogenous application of the naturally occurring auxins and synthetic homologues was found to induce the cell elongation through the membrane potential modification by

activating the proton ATPase on the plasma membrane (Rayle and Cleland, 1992; Stirnberg *et al.*, 1995). Following the specific auxin-binding sites in the plasma membranes of the maize coleoptiles, the auxin-binding protein (ABP1) was identified and it was postulated to be the auxin receptor. The exogenous addition of the purified corn ABP1 on tobacco protoplasts conferred the ability of the protoplasts to respond to auxin, while antibodies raised against ABP12 blocked the auxin-induced hyperpolarization (Barbier-Brygoo *et al.*,1989). Antibodies against the putative form of auxin-binding site of ABP1 mimicked the auxin effect and membrane hyperpolarization was induced (Venis *et al.*,1992).

1.5.2 Cytokinins

In the induction of cell division cytokinins act synergistically with auxins. The cytokinin receptor has been identified by examining hypocotyls segments of calli grown in a media with different kinetin and NAA concentrations assisted in the identification of CRE1 as a cytokinin-insensitive phenotype in *Arabidopsis* (Inoue *et al.*, 2001) was found to be caused by mutations which are present in the CRE1 gene and on yeast the cytokinin-dependent growth phenotype was conferred by the expression of CRE1. The *cre*1 mutant was impaired in the cell division and differentiation which is important for proper root vascular tissue formation (Mahonen, 2000).

The genetic characterization of the cytokinin-independent (CKI) mutants has lead to the identification of the cytokinin receptor. The CKI1 gene in *Arabidopsis* has been found to be homologous to the receiver domains of the prokaryotic two-components regulators and histidine kinase (Kakimoto, 1996). This gene encodes for a 125 kDa protein product

which has two membrane-spanning sequences of hydrophobic amino acids at the N-terminal, followed by the histidine kinase domain and a receiver domain at the C-terminal. Schaller (1997) postulated that the region based on CKI1 polypeptide structure between those two hydrophobic domains are involved in the sensing of the cytokinin.

1.5.3 Gibberellin (GA)

The use of mutant analyses has allowed several intermediate genes involved in the GA signalling in a number of plant species to be identified. Two GA signalling intermediates which act as a negative regulator of GA responses, *SPINDLY (SPY)* (Jacobsen *et al.*, 1996) and *GA-INSENSITIVE (GAI)* (Peng *et al.*, 1997) have been cloned in *Arabidopsis.* By promoting the expression of such repressor GAI may act directly or indirectly to repress GA-induced genes transcription. The GA-mediated induction of gene expression has also been demonstrated in barley aleuronic cells (Jacobsen *et al.*,1995). Essential information on GA signalling intermediates including a second messenger called cGMP (Penson *et al.*, 1996) sugars (Perata *et a*l., 1997) was provided by the given experimental system. The exogenous application of GA on barley aleuronic cells affected the levels of the cellular signalling molecules (Bush, 1996) including the hydrolytic enzyme synthesis and secretions (Jones and Jacobsen, 1991). More evidence from other biochemical studies showed GA-induced elevation of cGMP levels (Penson *et al.*, 1996) and GA- induced elevations of cytosolic calcium (Bush, 1996) to early events in the GA signal transduction and links these two 2nd messengers.

1.5.4 ABA

ABA signal transduction studies which were done in the stomatal system which involves the orchestration of ion channel activities at the plasmalemma and tonoplast (Blatt and Grabov, 1997). This process appeared to rely on a performed system of ABA receptor(s), protein kinases and their antagonist, protein phosphatases (Pei *et al.*, 1997). Targets of this ABA signal pathway are formed by many different cation channels and anion channels. The second messenger cyclic ribose (cADPR) identification as ABA second messenger represents a breakthrough in the ABA signal research (Wu *et al.*, 1997). Microinjection technique was used to show cADPR's ability to induce ABA-dependent gene transcription modulated by Ca^{2+} and that NAD^{+} is a likely substrate for the ADPR cyclase. The cADPR activity targeted the ryanodine-sensitive calcium ion channels which have been localized at the vascular membrane tonoplast (Allen *et al.,* 1995; Muir and Sanders 1996). Therefore cADPR probably mediates the response by triggering calcium ions release into the cytosol.

1.5.5 Ethylene

Ethylene receptors and possibly osmosensors in *Arabidopsis* are histidine kinases. Studies have shown that the receptors of ethylene are homologous to the histidine kinases of the two-component signalling systems which have been observed in some eukaryotic and prokaryotic species and function in the second messenger cyclic AMP synthesis, osmoregulation and oxygen sensing (Bleecker, 1999). Mutants with lesions in ethyl-specific response such as *etr* 1, *ctr* 1 and *ein* 1 to *ein* 7 were isolated and some of the affected loci subsequently cloned (Ecker, 1995). The *etr* 1 mutant locus conferred

dominant ethylene insensitivity and encodes a membrane protein with homology to the two-component histidine kinases of yeast and bacteria (Chang *et al.*, 1993). Biochemical studies found that the gene product was an ethylene receptor with a diminished binding capacity for ethylene in the *etr* 1 mutant protein (Schaller and Bleecker, 1995). Therefore the ethylene signalling system consists of two components, the sensor consisting of a signal-input domain and a catalytic histidine kinase transmitter domain and the response regulator.

1.6 Effects of "classical" hormones on salinity, drought and cold stress responses

ABA plays a major role in drought, salinity and cold stress responses and multitude of genes are induced by water stress and thought to be involved in the plant stress tolerance and responses. In *Arabidopsis thaliana* four signal transduction pathways control these genes. The two of these four signal transduction pathways are ABA-dependent and the other two are ABA-independent. The Dehydration Responsive Element (DRE) is a *cis*-acting element participating in one of the ABA-independent signal transduction pathways and the protein that binds to this element has been characterized. Drought- and ABA-inducible MYC and MYB homologues are also involved in the ABA-responsive gene expression in *Arabidopsis*. In addition a number of genes for protein kinases, enzymes involved in phosphatidyl inositol metabolism (PI turnover) and transcription factors play a signalling role in water stress and they are thought to be participating in the stress signal transduction cascades (Shimazaki *et al.*, 1997).

In another study a chimeric gene construct was introduced in *Arabidopsis* plants consisting of the Firefly luciferase coding sequence (LUC) under the control of the stress-

responsive RD29A promoter. The activity of LUC in the transgenic plants characterized in *vivo* using luminescence imaging system showed the endogenous RD29A gene expression. A large number of mutants with constitutive expression of osmotically responsive genes (*cos*), low expression of osmotically responsive genes (*los)* and high expression of osmotically responsive genes (*hos*) were identified. The *los* and *hos* mutants were grouped into 14 classes according to their defect in response to one or a combination of stress and ABA signals. Based on these classes a model for stress signalling in higher plants was proposed and it is contradicted belief that ABA-dependent and ABA-independent stress signalling pathways act in a parallel manner, the data revealed that these pathways cross-talk and converge to activate stress gene expression (Ishitani *et al*., 1997).

In relations to Gibberellin and kinetin hormones, studies have shown that their application to *Suaeda fruticosa* (L.), plants grown in saline conditions (200 to 400 mol m(-3) NaCl) alleviated some of the inhibitory effect of salinity at 800 mol m (-3) on the shoot growth while the growth of the root was promoted by kinetin. In another study application of Gibberellin (GA) and/or cytokinin (CK) to the root of *Sorghum bicolour* (L.) *Moench* modified the growth rate of shoot, the adventitious and seminal roots in the young seedlings. For each population treated with hormones, the link between the weight of these organs were analysed and these links build a network of relationships called meristem network. Treatments with 1-100 µM CK modified the growth of the organs and the meristem network. But treatments with 1-100 µM GA modified the growth slightly and the meristem network was considerably changed. Application of CK and GA simultaneously resulted with growth similar to that of CK-treated plants and the meristem

network was similar to that of GA-treated plants. Similar experiments were performed on plants in saline conditions of 150 mM NaCl. Both the growth and meristem networks were modified by salinity. Growth was unrelated to meristem network variations in non-salinized plants, while an optimal level of relationship for growth was observed in salinized plants. The result showed that the stress generates an imbalance between the self-stimulation of meristem activity and its regulation by other organs. (Amazallag, 1999).

Other studies have shown that under different levels of both chloride (Cl^-) and sulphate (SO_4^{-2}) dominated types of salinity seedlings of the salt sensitive wheat cultivar C-306 evolved more ethylene than the salt tolerant cultivar *Kharchia*-65. Pre-sowing seed soaking treatments with plant hormones kinetin, gibberellin and to a lesser extend with indole-3-acetic-acid (IAA) alleviated the effect of the salt as apparent from the seedlings dry mass. Under both saline and non-saline conditions seedlings treated with hormones evolved more ethylene. Moreover under both types of salinity ABA inhibited production of ethylene and the growth of seedlings (Datta *et al.*, 1998).

1.7 Polypeptide plant hormones

The polypeptide hormones were previously thought to be present only in animals and yeast. The discovery of systemin, an 18-amino acid polypeptide defence hormone demonstrated the existence of this class of hormones in plants (Pearce *et al.*, 1991). Other peptides hormones that have been characterized in plants include, ENOD40 and phytosulfokines. More recently, peptide hormones have been found in plants and

immuno-analogous plant natriuretic peptide (irPNP) is one example. Since irPNP is relevant to the study this hormone will be reviewed.

1.8 Natriuretic peptides hormones

Animal natriuretic peptide (ANP) is a peptide hormone which is synthesized in the cardiac atria in mammals and it is involved in the regulation of blood pressure and extravascular fluid volume (DeBold, 1985; Lang *et al.*, 1985). In animals, the natriuretic peptide system has been identified in the fish gills (Toop *et al.*,1995 a,b; 1998 Donald *et al.*,1994; Donald *et al.*,1997; Sakaguchi *et al.*, 1993), in the heart (Cerra *et al.*, 1992; Donald *et al.*, 1992) in the brain (Donald *et al.*, 1992; Suzuki *et al.,* 1992) and in the kidney (Toop *et al.*, 1995b). In plants, NP-like molecules have been identified in roots, stems, leaves and flower petals, even though there was variation in the total content of NP-like molecules between organ and species (Vesley *et al.*, 1991; Vesley *et al.*, 1993).

The use of high performance gel permeation chromatography (HPGPC) on extracted pro-ANF and ANF analogues from plants indicated that there was similarity between putative pro-ANP and ANP analogues isolated from plants with molecular weight of approximately 3.1 kDa (Vesley *et al.*, 1993). Moreover it was also shown using immunoaffinty studies that rabbit anti-human ANP (human canine) antiserum successfully purified NPs from ivy (*Hedera helix*) leaves (Billington *et al.*, 1997). The purified eluted irPNP fraction was further perceived to be consisting of several protein species having molecular weight in the ranges of 3-4 kDa, 6-8 kDa and 9-16 kDa (Pharmawati *et al.*, 1998b). The lower molecular weights are comparable with the NPs

found in animals having molecular weight of approximately 3 kDa, the larger molecular weights could be interpreted as precursor analogues

1.8.1 Natriuretic peptide-dependent signals

Natriuretic peptide in animals are mediated via three types of receptors namely natriuretic peptide receptor A (NPR-A), natriuretic peptide receptor B (NPR-B) and natriuretic peptide receptor C (NPR-C). NPR-A and NPR-B are transmembrane proteins having an extracellular ligand binding domain, a single transmembrane domain, a protein kinase-like and guanylate cyclase (GC) intracellular domain and NPR-A is also known to be membrane-bound form of guanylate cyclase (guanylate cyclase-A or GC-A) (Garbers and Lowe, 1994). GC can be stimulated by internal or external stimuli such as nitric oxide (NO) and by natriuretic peptides such as rat ANP (rANP) through enzyme activation leading to the formation of a second messenger (Elliot, 1997). ANP and BNP (a type of natriuretic peptide found in animals) binding to the GC-linked receptors stimulates guanosine triphosphate (GTP) hydrolysis leading to the production of the second messenger cyclic 3'5'-guanosine monophosphate (cGMP) (Koller *et al.*, 1992; Maack, 1992). CGMP mediates in the induction of natriuresis and diuresis in the kidney and glands and inhibits the production of aldosterone in adrenal glands.

The third receptor NPR-C which lacks intrinsic GC activity was originally believed to be a clearance receptor that binds internalized degraded circulating natriuretic peptides (Levin, 1993; Maack, 1992). Recent studies have shown that NPR-C has been implicated in second messenger pathways which are independent of cGMP but dependent on cAMP. NP activated NPR-C has been shown to stimulate G-protein binding, which resulted in

decrease of cAMP levels and an increase of IP3 production respectively. This occurred by the inhibition of adenylate cyclase activity or stimulation of phospholipase C activity (Anand-Srivastava and Trachte,1993).

1.8.2 Functions of Natriuretic peptides

In animals the natriuretic peptides regulate water and salt balance in the heart, kidney, vascular tissue and adrenal gland (Koller and Goeddel, 1992). In plants, several ions transport systems have been studied in relations to NPs. NPs are involved in potassium (K^+), calcium (Ca^{2+}), sodium (Na^+), chloride (Cl^-) ions regulation and water transport. They are also involved in cGMP synthesis which in turn modulates ion transport proteins including calcium-activated K^+ channels, ATP-sensitive K^+ channels, inwardly $I_{(k^+ in)}$ and outwardly rectifying K^+ $I_{(k+ out)}$ channels and water channels (Kourie and Rive, 1999). In rat messangial cells addition of ANP at concentrations between 400 pm and 300 mM were found to hyperpolarize plasma membrane (Vm) significantly (Cermak *et al.*, 1996). The hyperpolarization was due to the increase in K^+ conductance. Furthermore the K^+ channels modulation by ANP was postulated, and it was suggested that ANP acts via NPR-A alters K^+ homeostasis through calcium-activated K^+- conductive pathway. In another study it was found that NP modulated K^+ channels. For example, the addition of hANP was found to increase K^+ channel conductance in *Xenopus laevis* oocytes (Miledi *et al.*, 1989).

In plants exogenous addition of rat ANP and irPNP induced stomatal pore aperture in *Tradescantia albiflora* in a concentration dependent manner (Gehring *et al.*, 1996; Pharmawati *et al.*, 1998 a, b). Some evidence has suggested that NPs are also involved in

homeostasis in plants. The exogenous ANP addition (pro-0ANP) at 5.9 pg/ml, increased the rate of the flow of solutes in carnations (*Dianthus caryophillus*) and *Chrysanthemum morifolium* stems and increased transpiration in cut-red maple leaves (Vesley *et al.*, 1993). In another study using *Tradenscantia multiflora* shoots it was found that there was a significant increase of radial water movement out of the xylem in the presence of rANP and irPNP treatment (Sumastika and Gehring, 1998).

1.9 The second messenger cGMP

Essential second messengers Ca^{2+}, cAMP, cGMP, IP3 and 1.2-diacylglycerol have been studied in animal systems (Nishizuka, 1984; Berridge and Irvine, 1994). Recently cyclic GMP has also been found in plants (Brown *et al.*, 1989; Bowler *et al.,* 1994; Hoshi, 1995). Aleurone layers and protoplasts which were isolated from barley grain contained cGMP (Penson *et al.*, 1996). Pharmawati *et al.*, (1998b) using radio immunoassay, found that there was cGMP present in the root stele of maize and that the level rapidly and significantly increased in response to irPNP.

Cyclic 3'5'-guanosine monophosphate (cGMP) is a second messenger which induces natriuresis and diuresis in the kidney and inhibits aldosterone production in the adrenal gland. Synthesis results from the binding of ANP and BNP to GC-linked receptors which stimulates GTP hydrolysis leading to the formation of cGMP. As discussed above it is also involved in signal transductions of both the "classical" and the "new" types of hormones.

Cyclic GMP activity can be inhibited or stimulated by internal or external stimuli via enzyme activation. In animals the function of guanylate cyclase-cGMP enzyme which catalyzes cGMP formation from GTP could be stimulated with Nitric oxide (NO) and atrial natriuretic factor (ANF is the term previously used for ANP). In plants, stimuli such as gibberellic acid treatment of barley aleurone, light stimulation of bean or nitric oxide (NO) treatment of spruce needles caused transient increase in cGMP levels (Penson *et al.*, 1996; Pfeiffer *et al.*, 1994). Cyclic GMP is also involved in photo-morphogenesis, in light-mediated signal transduction and ion channel regulation (Bowler *et al.*, Brown *et al.*, 1989; Penson *et al.*, 1996) and in phytochrome-mediated signal transduction via Ca^{2+} uptake by darkened oat protoplasts (Sokovolovsky *et al.*, 1996). In other studies exogenous application of cGMP stimulated the opening of the stomata through Ca^{2+} signalling in *Commelina communis* L and the maximal opening of the stomata was reached at 50μM 8-Br-cGMP (Cousson and Vavasseur, 1998) and *in Tradescatia* sp maximal opening of the stomata was reached at concentration of 100μM (Pharmawati *et al.*, 1998a). Methylene blue and LY 83583 (which are potent inhibitors of guanylate cyclase) or BAPTA (which is an inhibitor of intracellular Ca^{2+} release) reversibly inhibited this induction. More studies showed that 10 μM of exogenous 8-Br-cGMP significantly reduced H^{+}-ATPase activity measured as inorganic phosphate (Pi) release in stem or leaf tissue microsomal of *Tradescantia* (Suwastika and Gehring, 1999) as well as in microsomal membrane from maize root stele (Pharmawati *et al.*, 1999).

1.10 Immunoreactive plant natriuretic peptide (irPNP)

IrPNP is a natriuretic plant peptide which has been isolated and purified by immunoaffinity from ivy leaves (*Hedera helix*) (Billington *et al.*, 1997). This purified irPNP has been shown to induce transient cGMP elevation levels (Pharmawati *et al.*, 1998b) and to cause immediate net H^+ influx and delayed net K^+ and Na^+ uptake in *Zea mays* root stele tissue (Pharmawati *et al.*, 1999). In plants it has been observed that irPNP and rANP increased radial water movements significantly out of the xylem of shoots of *Tradescantia multiflora*. 8-Br-cGMP addition enhanced this increase (Suwastika and Gehring, 1998). Further studies showed that mercuric chloride ($HgCl_2$), sodium azide (NaN_3) and guanylate inhibitor LY 83583 inhibited the water movements suggesting that NP effects might be mediated via guanylate cyclase-dependent regulation of water channels. In maize root and *Tradescantia* leaves membranes, rANP and irPNP have been identified to be involved in H^+- ATPase activity as by proton pumping and Pi release (Pharmawati *et al.*, 1999; Suwastika and Gehring, 1999). Exogenous irPNP and rANP are also involved in the stomatal movement. They induced the stomatal pore aperture in *Tradescantia albiflora* in a concentration dependent manner (Gehring *et al.*, 1996; Pharmawati *et al.*, 1998 a,b). The stomatal opening induction by rANP and kinetin was inhibited by two guanylate cyclase inhibitors which are 10 µM methylene blue (MB) and 20 µM LY 83583 (Pharmawati *et al.*, 1998b). The stimulation was diminished by the presence of ABA. This study suggested that rANP action in the opening of the stomata is guanylate cyclase-dependent or operates through guanylate cyclase up-regulation. Since both rANP and irPNP have been shown to increase stomatal pore aperture it is suggested that NPs in plant systems may be involved in controlling movement of water to other

cells and tissue. However further work needs to be done in order to elucidate the molecular mechanism of NP function in plants.

2. Materials and Methods

2.1 Sterilisation of seeds

Arabidopsis thaliana seeds were put into a sterile 1.5 ml eppendorf tube in 500 µl of 95% absolute ethanol was added. The seeds were mixed with gentle shaking for 45 seconds. The seeds were allowed to settle and the ethanol was discarded. Seeds were rinsed three times with 1000 µl sterile distilled water and incubated in 1000 µl sterilizing solution (2 ml 100% bleach solution and a drop of Tween-20 in 17 ml sterile distilled water) for 10 minutes with gentle shaking in between each rinse. The sterilizing solution was discarded and the seeds were rinsed five times with 1 ml sterile distilled water. The seeds were finally placed on a sterile filter paper in a petri dish and left to dry in a laminar flow hood.

2.2 Seed germination assay

Seeds were transferred to germination media (1/2 MS10) containing different salt concentrations with and without 10 µM kinetin. The salt concentration ranged from 0 mM to 200 mM NaCl. Seeds were transferred to a 12 welled micro titer plate. Ten sterilized *Arabidopsis* seeds were transferred to each well containing the respective salt concentration with and without 10 µM kinetin using sterile tweezers. The micro titer plates were sealed with parafilm and placed at 4 °C for four days. After four days the microtiter plates were transferred to a 25 °C growth room under alternating light and dark.

2.3 Preparation of plants for the Atomic absorption spectrophotometer

After 24 days the plants were out rooted from each well of the micro titer plates, placed in sterile 1.5 ml epperndorf tubes and weighed (Figure 3). In each epperndorf tube 100 µl

of sterile distilled water was added together with the plants and then placed into an ice bucket containing liquid nitrogen for 10 minutes. The epperndorf tubes containing the respective samples were placed into a heating block at 90 °C for 10-15 minutes. The freezing and heating was repeated three times. Each sample was transferred to a 50 ml volumetric flask and the flask was filled with sterile distilled water. A Pye UniCam Solar M Series Atomic Spectrophotometer was used to determine the concentration of total potassium [K] and total sodium [Na] in the plant material (Allen *et al.*, 1995).

2.4 Sequence analysis of *Arabidopsis thaliana* and *Erucastrum strigosum*

Sequence analysis was performed using programs in the DNAMAN V4.0 (Lynnon Biosoft, Canada) sequence analysis package. Amino acid identities and similarities between protein sequences were determined using DNAMAN V4.0 (Lynnon Biosoft, Canada). Multiple sequence alignments were conducted using the Multiple Alignment component of DNAMAN V4.0. Phylogenetic analysis was conducted using the Clustal W program (Thompson *et al.*, 1994) and the results were rendered using the TreeView software package (Page, 1996).

2.5 Protein extraction

Erucastrum strigosum leaves treated with different salt and kinetin concentrations (G. Ngxaba, personal communication) were weighed in 50 ml falcon tubes. Using scissors the leaves were cut into small pieces into a mortar and ground into a powder under liquid nitrogen. The powder was removed using a spatula and transferred into 250 ml volumetric flask. For 10g of tissue, 50 ml extraction buffer (50 mM KCl, 1 mM EDTA, 10 mM Tris-HCl pH 7.4) and 50 ml methanol were mixed and added to the powder. The

mixture was stirred continuously overnight on the magnetic stirrer at 4 °C. The extract was filtered through filter paper/ muslin and a funnel into a 250 ml round bottom flask and placed on to a rotary evaporator (EYELA TOKYO RIKAKIKAI) for approximately 4 hours. To the dried mixture 50 ml of extraction buffer was added and placed at -70 °C for two days after which the mixture was freeze dried for two days. To the dried mixture 1ml sterile distilled water was added, transferred to a 1.5 ml epperndorf tube and centrifuged for 15 minutes at 13.2 rpm. The supernatant was transferred to another sterile 1.5 ml epperndorf tube and stored at -20 °C.

2.6 IrPNP Purification using BIOCAD Sprint system

The irPNP was affinity purified on a POROS 20 AL anti-ANP affinity column using the BIOCAD SPRINT system. The column was prepared as follows: Rabbit anti-ANP antiserum (Auspep, Parkville Australia) was re-suspended in 100 ml coupling buffer (0.1M HEPES, pH 7). An aliquot of the resuspended rabbit anti-ANP antibody (55 ml) was added to 0.8g POROS 20 AL resin (PerSeptive Biosystems, Framingham, USA) in 5 ml coupling buffer and rotated at room temperature for 8 hours. The resultant Schiff's base was reduced by adding 5 mg $NaBH_4$ per ml bed volume (11.5 mg) to the coupling solution and rotated for a further 2 hours at room temperature. The coupling mixture was centrifuged at 13 000 rpm (Epperndorf bench top centrifuge 5415D) for 1 minute at room temperature and the supernatant discarded. Residual aldehydes were quenched by adding 1.5 ml 0.2 M Tris buffer containing 11.5 mg $NaBH_4$ to the pelleted resin and rotated at room temperature for 2 hours. The POROS 20 AL anti-ANP affinity resin was packed into a POROS PEEK column (4.6 mm x 100 ml, 1.7 ml) and washed with 10 column volumes of equilibration buffer (1mM Tris/HCl, pH 7.5). To quantitate the level of irPNP

in the plant extracts, 50 ml was applied to the POROS 20 AL anti-ANP affinity column. The column was washed with 24 CV (column volumes) equilibration buffer and the bound protein eluted with 5 CV equilibration buffer containing 1M NaCl before re-equilibrating the resin with 15 CV equilibration buffer. Aliquots (1 ml) were collected at a flow rate of 10 ml/min. The peaks were integrated and the area under each peak calculated using the BIOCAD chromatogram analysis software.

2.7 Protein concentration determination: Bradford Assay

Protein concentration of extractions was determined according to the Bradford method (Bradford, 1976) using Bovine Serum Albumin (BSA) (Sigma) as a protein standard. BSA was used to construct a standard curve from 0 to 0.6 mg protein/ml (Figure A1, Appendix)

Twenty µl of a BSA standard was added to 200 µl of Bradford Reagent per well in micro titer plate. Absorbance was recorded at 620 nm on a Multiskan BICHROMATIC (Labsystems, Finland) plate reader and a standard curve constructed. Where samples were being assayed for protein concentration, 20 µl of the sample was added to 200 µl of Bradford Reagent. Appropriate blanks consisting of the relevant buffers were used and all assays were performed in duplicate.

2.8 Protein detection 15% SDS gel

2.8.1 Preparation of resolving gel

To a 50 ml falcon tube, 2.4 ml sterile distilled water, 5 ml 30% degassed Acrylamide/Bis (Bio-Rad, USA), 2.5 ml gel buffer (1.5 ml Tris-HCl, pH 8.8); 0.1 ml 10% w/v SDS; 50 µl 10% APS; 5 .l ml TEMED were added, gently swirled and loaded on to the Mini-

PROTEAN 3 casting stand (Bio-Rad, USA). Immediately 500 µl of sterile distilled water was added to the gel and left to polymerize for 45 minutes. The water was removed using a blotting paper.

2.8.2 Preparation of stacking gel

To 50 ml falcon tube 6.1 ml sterile distilled water; 2.5 ml 0.5M Tris pH 6.8; 1.3 ml 30% degassed Acrylamide/Bis; 0.1 ml 10% SDS; 50 µl 10% APS; 5 µl TEMED were added and mixed. The mixture was loaded onto the resolving gel and left for 30 min.

2.8.3 Sample preparation

To 1.5 ml epperndorf tube, 60 µl sample buffer and 3.16 µl 2-mecarptoethanol were added and mixed. The mixture was aliquoted to the samples and tubes prior to loading the samples were heat treated for 5 minutes at 100 °C. The gel was run at 200V for 35 minutes in1 x running buffer (25 mM Tris, 192 mM Glycine, 3.4 mM SDS).

2.9 Silver staining of the Gel

After electrophoresis, one gel was silver stained and on the other gel a Western blot was performed. The gel was removed from the cassette and placed in a clean staining container and was rinsed briefly with sterile distilled water. The gel was fixed in 100 ml of fixative solution containing 40% ethanol and 10% acetic acid, for 20 minutes with gentle rotation. The fixative solution was discarded and the gel was washed in 30% ethanol for 10 minutes. The ethanol was poured off and the washed gel was incubated in 100 ml of Sensitizing solution (Invitrogen life technologies, UK) for 10 minutes. After incubation, the Sensitizing solution was discarded and the gel was washed with 100 ml of 30% ethanol for 10 minutes followed by washing of the gel in 100 ml of sterile distilled

water for 10 minutes with gentle rotation. Immediately the gel was incubated in 100 ml of staining solution (Invitrogen life technologies, UK) for 15 minutes. After staining was complete, the staining solution was poured off and the gel was washed with 100 ml of sterile distilled water for 20-60 seconds. For the development of the gel, the gel was incubated in 100 ml of the developing solution for 4-8 minutes until bands started to appear and desired band intensity was reached. Once the appropriate staining intensity was achieved, immediately 10 ml of Stopper was added directly to the gel still immersed in the Developing solution and gently agitated the gel for 10 minutes. Once the colour changed from pink to colourless indicating that the development has been stopped the Stopper solution was discarded and the gel was washed with 100 ml of sterile distilled water for 10 minutes.

2.10 Mass Spectroscopy

Purified proteins were analyzed on a MALDI TOF mass spectrometer (Voyager-DEBiospectrometry Workstation, PerSeptive Biosystems) to determine the molecular mass of the isolated proteins. The MALDI-MS was fitted with a nitrogen UV laser (337 nm), and the matrix used was Sinapinic Acid (10 mg/ml) with 50% acetonitrile, 3% TFA in deionised water as solvents. Analysis was performed by Prof. W. Brandt (Dept. of Biochemistry, UCT) as per manufacturers instruction.

2.11 Western Blot

After electrophoresis, the gel was put in gel cassettes. The set-up was as follows: filter pad, filter paper, gel, nitro-cellulose membrane, filter paper and filter pad. After the set-up was put together it was placed in the running tank. The Bio-ice and magnetic stirrer

bar were put inside the running tank together with the transfer buffer (48 mM Tris, 39 mM Glycine, 20% Methanol) and the gel was run over night at 30V, 90 mA on the magnetic stirrer.

2.11.1 Blocking the membrane

After electrophoresis, the membrane was transferred into a container with a Blocking solution (5% non-fat dried milk, 0.1% (v/v) Tween 20 (Merck, Germany Cat no. 822184) and left for an hour at room temperature on an orbital shaker. The blocking solution was poured out and the membrane was rinsed twice for 2 minutes with TBS-T.

2.11.2 Primary antibody incubation

10 µl of rabbit anti-ANP antibody (Auspep, Parkville Australia) was diluted with 50 ml of TBS-T and was poured on to the membrane, placed on to the orbital shaker at room temperature for an hour. The membrane was briefly rinsed with two changes of washing buffer (TBS-T) followed by the 15 minutes washing in TBS-T for 15 minutes. The membrane was then rinsed three times with TBS-T for 5 minutes.

2.11.3 Secondary antibody incubation

5 µl of the Goat secondary antibody was diluted with 50 ml TBS-T and was poured on to the membrane and left for an hour on the orbital shaker. The solution was discarded and the membrane was briefly rinsed with two changes of TBS-T and then washed in TBS-T for 15 minutes. The membrane was then rinsed three times for 5 minutes with TBS-T.

2.11.4 Detection

Detection solutions A and B (Amersham Pharmacia Biotech, UK) were mixed in a ratio of 40:1. The excess washing buffer (TBS-T) was drained from the washed membrane and placed protein side up on SaranWrap ^{Tm}. The mixed detection reagents were pippetted on to the membrane incubated for 5 minutes at room temperature. Thereafter the excess detection reagent was drained off the membrane. The blot was placed protein side down on to a fresh piece of saranWrap^{tm}, wrapped up and the air bubbles were gently smoothed out. The wrapped blots were placed protein side up in an x-ray film cassette and a sheet of autoradiography film was placed on top of the membrane. The x-ray film was exposed for approximately 30 seconds to 2 minutes until the desired band intensity was reached. The film was then removed and replaced with a second sheet of unexposed film. The first piece of film was immediately developed and on the basis of its appearance the second piece of film was exposed for 2 minutes.

2.12 *Erucastrum strigosum* RNA extraction

Ten seeds of *Erucastrum strigosum* were sown in each pot that were filled to the top with silica sand treated with 10 ml of 1M Murashige and Skoog Basal (Sigma, USA, Cat. No. M-5519) media and 40 ml tap water. The plants were placed in a growth room at 25 °C under alternating light and darkness, were watered twice a week with a 50 ml (20g: 5000 ml) full-strength chemicult (Chemicult, SA) and 50 ml tap water alternately. After 28 days the plants were divided into two per pot and kinetin-salt treatment was commenced at 11:00 am each day and only 0.2 g of leaves were harvested at 14:00 pm the following day for RNA extraction using RNeasy Plant Mini kit (50) Qiagen, USA, Cat. No. 74904.

The kinetin (Sigma, USA, Cat. No. K-0753) concentrations was at 0 and 4 µM. The NaCl concentration used was increased by 100 mM of NaCl per day to obtain 0, 200, 300, 400 mM concentrations respectively. The RNA extracted was quantified using a spectrophotometer at $OD_{260\ nm}$ (Milton Roy Co, USA, Cat. No. 33600800).

2.13 Preparation of the DIG labelled Probes

The *Arabidopsis thaliana* genomic DNA used was obtained from I. Ludidi (personal communication). Two primers were designed for the Dehydrin gene (exon 1) Accession No. X64199, the Forward Dehydrin primer: 5' GCTCTAGATCCCTATCCAAAGTTGC 3' and the Reverse Dehydrin primer: 5'TACTGCAGAGCTACTCCCAGAACG 3'. The Dehydrin gene was amplified using the Expand high fidelity PCR System. The PCR reaction (200 µl) was set up as follows: sterile distilled water (135 µl), 10X PCR buffer + 1.5M MgCl (20 µl), 10 mM ATP (4 µl), 10 mM CTP (4 µl), 10 mM GTP (4 µl), 10 mM TTP (4 µl), 10 µM Forward Dehydrin Primer (6 µl), 10 µM Reverse Dehydrin Primer (6 µl), *Arabidopsis thaliana* DNA 600ng (12 µl), Taq DNA polymerase (5 µl) (Roche, Germany, Cat. No. 1732641) and amplified as follows: 95 °C 2 min (1 cycle), 95 °C 30 seconds (30 cycles), 55 °C 30 seconds (30 cycles), 72 °C 50 seconds (30 cycles), 72 °C 5 minutes (1 cycle), 4 °C 10 Minutes (1 cycle). The PCR product was purified using the MinElute PCR purification Kit (50) Qiagen, USA Cat. No. 28004.

The *Arabidopsis thaliana* PNP-A gene (Accession number: AC005724) was previously cloned into pBluescript (pSkatPNP) vector (R. Bastian, personal communication). The plasmid, pSkatPNP 10 µg was digested with Bam HI(10 U/µl) and Hind III(10 U/µl) overnight at 37 °C. Subsequently, the reaction mixture was run on a 0.8% agarose gel at

80 V and the *Arabidopsis thaliana* PNP-A band (368 bp) excised and purified using the QIAquick Gel Extraction kit (50) Qiagen,USA Cat. No.28704.

2.13.1 DIG labelling of the Probes

To two 1.5 ml sterile epperndorf tube, 4 µl of the purified Dehydrin PCR product was added and into the other tube, 4 µl of purified PNP-A gene was added. To each tube 12 µl sterile distilled water, 4 µl DIG-Nick translation mix (Roche, Germany (Cat. No. 1745856) were added and the reactions were left at 15 ° C for 90 minutes. The reactions were stopped by chilling to 0°C for 5 minutes followed by the addition of 1 µl 0.5M EDTA. The DiG labelled probes were stored at -20 °C.

2.13.2 Quantitation of the DIG-DNA Probes

Serial dilution of the DiG labelled probes was carried out according to the Roche Molecular Biochemical DIG Application Manual for filter Hybridization. From each required serial dilution 1 µl was taken and spotted onto a nylon membrane (Hybond N^+) Amersham, Pharmacia Biotech, UK). Fixation of the DNA on to the membrane was achieved by exposing the membrane under UV light for 5 minutes. Immediately the membrane was washed briefly with the washing buffer (Maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl) and 0.3% Tween-20) and was incubated in the blocking solution (maleic acid buffer with 1% non-fat milk powder) for 30 minutes at room temperature on orbital shaker. Thereafter the membrane was incubated with Anti-DIG alkaline phosphatase (1:5000 dilution in blocking solution) antibody solution for 30 minutes at room temperature covering. The antibody solution was discarded and the membrane was washed twice, 15 minutes per wash in washing buffer at room temperature. For detection,

the membrane was placed in the detection buffer (0.1 M Tris, 0.1M NaCl) for 2 minutes. The detection buffer was poured off and the colour substrate solution (45 µl NBT (75 mg/ml), 35 µl BCIP (50 mg/ml) solutions in 10 ml detection solution) was added. The membrane was placed in the dark to allow the colour development to occur and was allowed to continue for approximately 16 hours. When the spots appeared in sufficient intensity, the reaction was stopped by washing the membrane with sterile distilled water for 5 minutes. To estimate the concentration of the experimental probes the spots intensities were compared to the control.

2.14 Northern Blot

The Northerns were carried out according to the Roche Molecular Biochemical DIG Application Manual for filter Hybridization. Only the following change was made; For the prehybridization and the hybridisation, the temperature was changed from 68 to 50 °C for the DNA probe that was used.

2.15 Slot Blot

The Slot blots were carried out according to the Bio-Rad Bio-Dot® SF Microfiltration Apparatus Instruction Manual (Bio-Rad, USA (Cat. No 170-6542/3).

3. RESULTS AND DISCUSSION

One of the problems facing agriculture today is soil salinity as this places constraints on food production and limits the use of previously cultivatable land. This has led to research with the aim of breeding crop cultivars with improved salt tolerance. Salinity stress or salt stress is primarily caused by high sodium ions (Na^+) and chloride ions (Cl^-) concentrations in the soil solutions which impact negatively on the critical biochemical processes (Wyn, 1981). Salt movement into roots and shoots results from the transpirational flux which is required to maintain water in the plant (Hasegawa *et al.,* 1994; Yeo, 1998). Unregulated transpiration can result in accumulation of ions in the aerial part of the plant which is toxic to the plant (Munns and Termaat, 1986; Yeo, 1998). The accummulation of ions in mature and old leaves which then dehisce have also been observed under salt stress (1992; Munns, 1993).

Cytokinins are a class of plant hormones which are involved in the stimulation of the plant cell division, lateral and shoot induction, retardation of senescence (Westhoff *et al.,* 1998). Kinetin, although not identified in plants, is the most biologically active cytokinin. Transportation of cytokinins in plants from the roots to the leaves takes place in the phloem pathways and reaches all the cells present in the plant by diffusion (Kambo *et al.,* 1998). We therefore sought to ask how salt and salt-kinetin treatment affected the germination of *Arabidopsis thaliana* seeds.

3.1 Seed germination of *Arabidopsis thaliana* treated with NaCl with and without kinetin

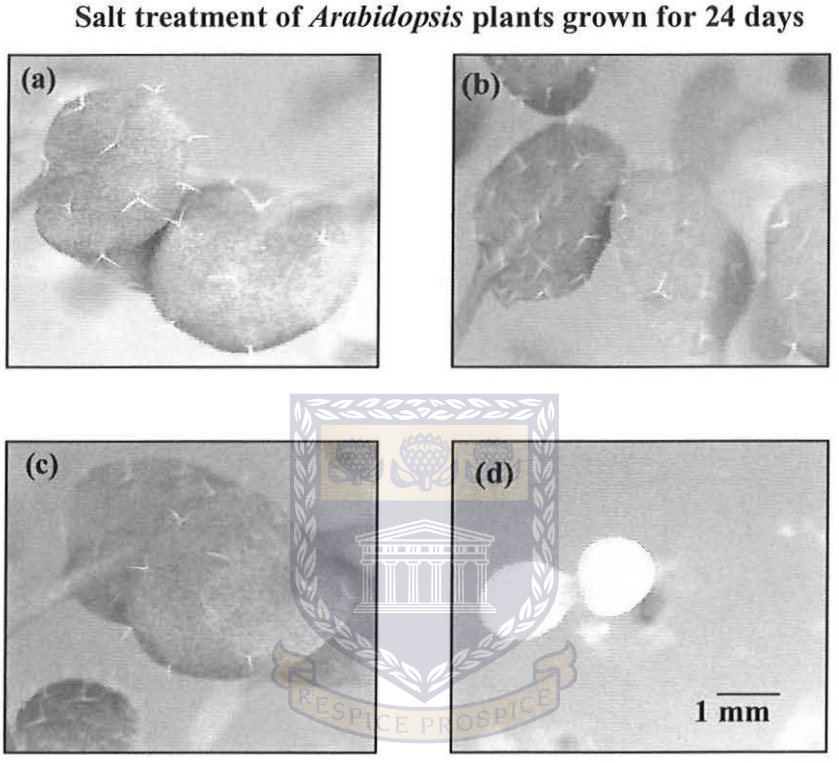


Figure 1: Germination assay of *Arabidopsis thaliana* (cv: Columbia) seeds treated with different concentrations of salt. *A. thaliana* seeds were placed onto media treated with different salt concentrations and left for 24 days in which time growth was monitored. (a) 0 mM NaCl; (b) 50 mM NaCl; (c) 100 mM NaCl; (d) 150 mM NaCl.

To determine the effect that salt has on the *Arabidopsis thaliana* plants, seeds were germinated on media treated with different salt concentrations (50, 100, 150, 200) mM NaCl as discussed in 2.2 section for 24 days. The plants had salt tolerance to a certain extent as indicated in Figure 1 (a), (b) and (c) where the seeds were germinated on media treated with 0 mM NaCl, 50 mM NaCl and 100 mM NaCl respectively. The leaves size,

colour, leaf number and shoot height, which was 10 mm for all the plants, were not affected by these specific salt treatments (Figure 1 and Figure 3). As the salt concentration was increased to 150 mM NaCl the plant salt tolerance mechanism was greatly affected (Figure 1(d)). The leaves turned yellow and the plants became stunted with a shoot height of 5mm and the percentage growth of the plants was decreased from 80% to 60% (Figure 1(d) and Figure 3). At 200 mM NaCl treatment the plant growth was severely affected with a shoot height of only 3 mm and the number of plants that survived was reduced to 40% (Figure 3).

Kinetin treatment of *Arabidopsis thaliana* plants grown for 24 days.

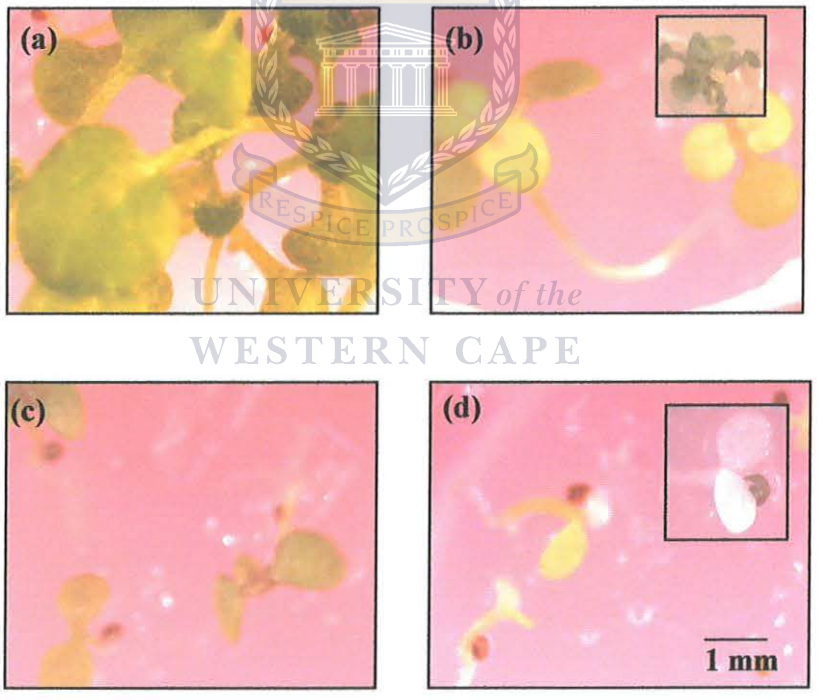


Figure 2: Germination assay of *Arabidopsis thaliana* (cv: Columbia) seeds treated with different concentrations of salt with kinetin. *A. thaliana* seeds were placed onto media treated with different salt concentrations and 10 □M kinetin (K) and left for 24 days in which time growth was monitored. (a) 0 mM

NaCl + 0 mM K with inset 0 mM NaCl + 10 µM K; (b) 50 mM NaCl + 10 µM K; (c) 100 mM NaCl + 10 µM K; (d) 150 mM NaCl + 10 µM K with inset 200 mM NaCl + 10 µM K

To determine the effect of salt-kinetin treatment on *Arabidopsis thaliana* plants, seeds were germinated on media treated with different salt concentrations (50, 100, 150, 200 mM) NaCl with 10 µM kinetin respectively as discussed in 2.2 section for 24 days. When comparing salt-treated and salt-kinetin treated plants it was found that plants grown on salt-kinetin treated media were more susceptible to salt than plants grown in only salt treated media (Figure 1 and Figure 2). The salt inhibition on plants germination started at 150 mM NaCl and reduced the number of plants germinated (Figure 2 (d)).

As the salt treatment was increased the plants became stunted as indicated by the reduction of the shoot height from 10 to 1 mm (Figure 3). At the 150 mM NaCl treatment, the salt-kinetin and salt treatment severely affected the height of the shoot (Figure 3).

The plant leaves were also severely affected by the 150 mM salt-kinetin treatment, which resulted in, the decrease of the leaves size and number (Figure 2 (c)). The colour of the plant leaves were also affected from 50 mM for salt-kinetin treatment (Figure 2 (b), (c) and (d)) when compared to salt treatment alone where colour was affected at 150 mM NaCl (Figure 1 (d)). The yellowing of the leaves indicated this colour change and in some cases the leaves turned white due to a bleaching effect (Figure 2 (d) inset). This indicates the accumulation of a high concentration of salt in the leaves.

To determine the physiological effects of salt and kinetin treatment on plants the untreated and treated plants were harvested for analyses.

	0	10 □M K	50		100		150		200	
	-	+	-	+	-	+	-	+	-	+
Germination after 24 days (in %)	85	37.5	80	70	70	70	60	30	40	20
Mean shoot height after 24 days (in mm)	10	10	10	8	10	5	5	3	3	1
Mean total fresh weight of shoot after 24 days (in mg)	23.6	8.35	32.9	6.1	23.6	4.9	2.3	1.5	1.4	1.5

Figure 3: Table for the analyses of germination, mean shoot height and total fresh weight of *A. thaliana* plants treated with different salt concentrations (0, 50, 100, 150 and 200 mM NaCl) with or without 10 µM kinetin (K) for 24 days. After 24 days the plants shoot height were measured the plants harvested and weighted. No Kinetin added: - ; 10 µM kinetin added: +.

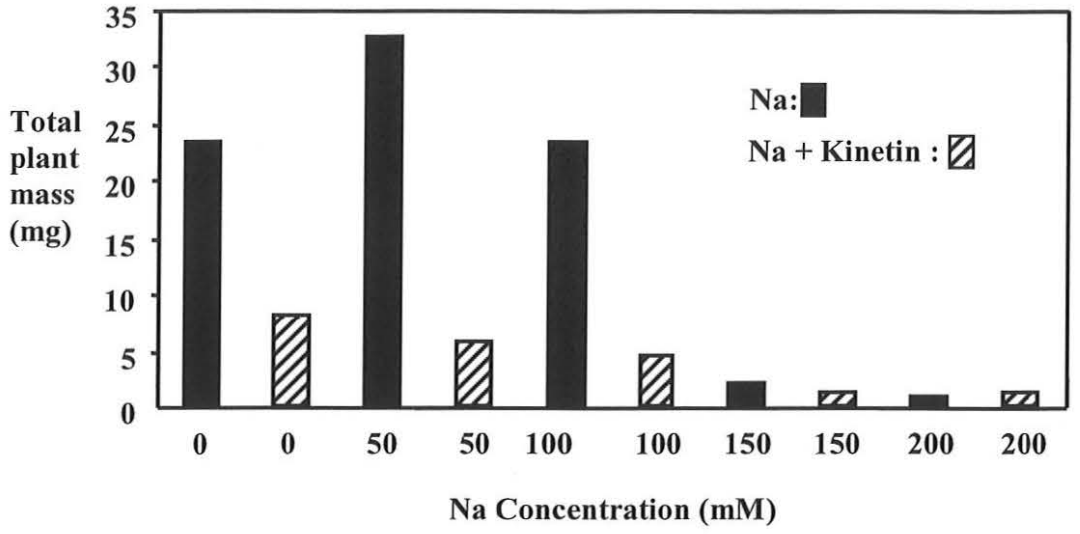


Figure 4: Graph of *A. thaliana* total plant mass (mg) versus NaCl (Na) concentration (mM) with and without 10 µM kinetin. *A. thaliana* plants were grown for 24 days on various NaCl concentrations (0, 50, 100, 150, 200) mM (black bars) with and without 10 µM kinetin (striped bars) after which they were harvested and weighed.

To determine the extent to which the salt and salt-kinetin treatment affected the plants, plants were harvested after 24 days, weighted and analysed. The salt-kinetin treated plants weighed less than the untreated, kinetin-treated and salt-treated plants (Figure 3 and Figure 4). At 50 mM and 100 mM NaCl treatments, plants masses were 32.9 mg and 23.6 mg respectively (Figure 3) indicating that plants could tolerate salt to a certain extent but as the salt was increased, at 150 mM and 200 mM NaCl, salt tolerance of plants was reduced resulting in the death of more plants and the subsequent lower mass values 2.3 mg and 1.4 mg respectively (Figure 3 and Figure 4). The number of plants that germinated was greatly affected by the salt treatment, the plant numbers reduced from

80% at 50 mM NaCl to 40% at 200 mM NaCl and the shoot height was also reduced from 10 mm to 3 mm respectively.

If this is compared to salt-kinetin treated plants at 50 mM and 100 mM NaCl, the masses were 6.1 mg and 4.9 mg respectively (Figure 3 and Figure 4). These values were lower than the salt treated plants at the same concentrations; this indicates that the salt-kinetin combination has an effect on the salt tolerance mechanism of plants making the plants more susceptible to salt. As the salt concentration was increased to 150 mM and 200 mM NaCl the masses were 1.5 mg and 1.5 mg respectively and the growth percentage was reduced from 70% at 50 mM NaCl + 10 µM kinetin to 20 % at 200 mM NaCl + 10 µM kinetin.

The kinetin treated plants weighed less than untreated plants, 8.35 mg and 23.6 mg respectively and the growth percentage was 37.5 % and 85% respectively (Figure 3). The shoot height was however not affected in both the treated and kinetin treated plants and remained at 10 mm (Figure 3). This indicates that the amount of kinetin added was toxic to the plants, independently of the salt treatment.

Kinetin is a plant hormone that regulates cell division and differentiation of the plant and is also involved in stomatal opening (Blackman and Davies, 1983; Marten *et al.,* 1991; Pharmawatti *et al.,* 1998a). Therefore, the opening of the stomata could have resulted in further accumulation of salt in the plants, which would explain the salt sensitivity of the plants treated with salt-kinetin. As indicated by the results in Figures 1, 2, 3 and 4, NaCl-kinetin treatment supports the observation that the transpiration flux becomes unregulated

(Munns *et al.,* 1986; Yeo, 1998) resulting in the accumulation of the salt in plant leaves, stunted growth and the reduction of the number of the plants grown. The NaCl-kinetin treatment enhanced the salt toxicity to the plant by causing the plants to be more susceptible to salt and thereby accelerating plant death. It has been reported that the salt effects on plants results from both water deficit leading to the osmotic stress and the effects of excess sodium ions on the key biochemical processes (Zhang and Blumwald, 2001).

We observed that *A. thaliana* seeds only treated with kinetin, 37.5 % germinated compared to untreated seeds (Figure 3). The resulting plants weighed less than untreated plants (Figure 3) though kinetin treated plants had well developed leaves (Figure 2 (a) inset). Exogenous application of cytokinins could have two opposite effects, it could increase the cytokinin content present in the plant cell by promotion of cytokinin biosynthesis or alternatively promote cytokinin degradation by increasing cytokinin oxidase activity. Cytokinins promote cell division, leaf expansion, accumulation of chlorophyll, conversion of etioplasts into chloroplasts and delay leaf senescence. It is possible that the latter could still have occurred when we applied kinetin exogenously resulting in accelerated plant senescence though the well developed leaves and normal shoot height could still have occurred independently.

3.2 Analysis of total potassium [K] and total sodium [Na] concentrations in *Arabidopsis thaliana* plants

Ion homeostasis in saline environments is dependent on transmembrane transport proteins that mediate ion fluxes, including H^+ translocating ATPases and pyrophosphatases, Ca^{2+} ATPases, secondary active transporters and channels (Niu *et al.,* 1995; Sze *et al.,* 1999). In hypersaline environments characterized by high NaCl concentrations, results in the disturbance of ionic steady state not only for Na^+ and Cl^- but also K^+ and Ca^{2+} (Niu *et al.,* 1995). External Na^+ negatively impacts intracellular K^+ influx reducing acquisition of this essential nutrient by cells and also high NaCl causes cytosolic accumulation of Ca^{2+} and this apparently signals stress responses that are either adaptive or pathological (Hasegawa *et al.,* 2000). Therefore our knowledge of how plants re-establish osmotic and ionic homeostasis after salt stress imposition and then maintain physiological and biochemical steady states necessary for growth and completion of life cycle in the new environment is fundamental to our understanding of plant salt tolerance.

To analyse the total potassium and sodium concentrations in untreated and treated *A. thaliana* plants, plants were harvested and prepared for the atomic spectrophotometer as discussed in 2.3 section. The plants were treated with different salt concentrations (0, 50, 100, 150 and 200) mM NaCl with and without 10 µM kinetin for 24 days after which they were harvested. The plants were snap-frozen and heat shocked to allow the release of ions from the plants. The resulting solution was ran through the atomic

spectrophotometer to determine the concentration of sodium and potassium ions present in the plants.

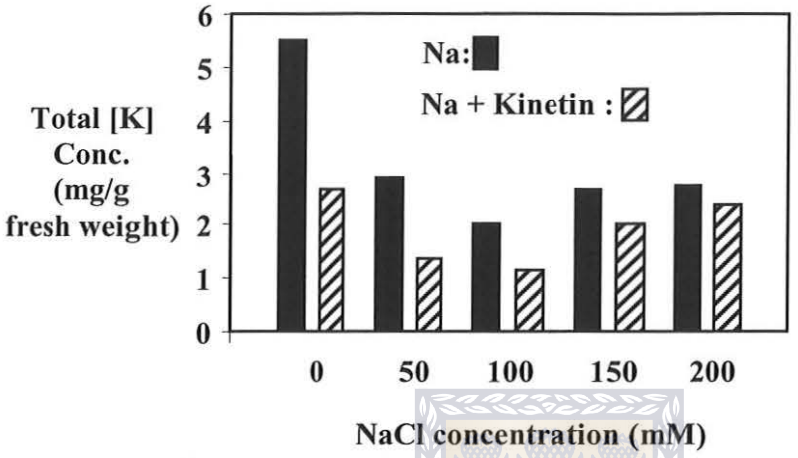


Figure 5: Graph of total potassium [K] concentration (mg/g fresh weight) versus NaCl concentration (mM) with and without 10 µM kinetin. *A. thaliana* plants were grown for 24 days on various NaCl concentrations (0, 50, 100, 150, 200) mM (black bars) with and without 10 µM kinetin (striped bars) after which they were harvested, treated and subject to ion analyses via a atomic absorption spectrophotometer.

In Figure 5 the untreated *A. thaliana* plants had the highest potassium concentration as compared to the plants treated only with 10 µM kinetin, salt or salt-kinetin. The untreated plants had total potassium concentration of 5.514 mg/g fresh weight (Appendix A1). When comparing the salt treated plants and the salt-kinetin treated plants, the salt treated had more total potassium concentration (Table A1). The 10 µM kinetin treated plants had total potassium concentration of 2.66 mg/g (Figure 5 and Appendix A1), nearly half the amount of the potassium concentration as compared to the untreated plants (5.514 mg/g). In *A. thaliana* plants treated with 50 mM + 10 µM kinetin and 100 mM NaCl + 10 µM

kinetin the total potassium concentrations were (1.397 mg/g and 1.176 mg/g) respectively, were approximately half amount of the total potassium concentration in plants treated only with salt at the same concentration (2.871 mg/g and 2.001 mg/g) respectively (Figure 5, Appendix A1).

The total potassium concentration was decreasing in both the salt and salt-kinetin treated plants at 0, 50 and 100 mM NaCl concentrations. As the salt concentration was increased to 150 mM NaCl in both salt and salt-kinetin treated plants the total potassium concentration increased as indicated by the values 2.707 mg/g and 1.99 mg/g respectively (Figure 5, Appendix A1). At the 200 mM NaCl treatment with and without kinetin the total potassium concentration further increased as indicated by the values 2.37 mg/g and 2.782 mg/g respectively (Figure 5, Appendix A1). As indicated by the graph in Figure 5 the total potassium concentration in untreated plants was high and there was a steady decrease at 50 mM and 100 mM NaCl treatments. It was previously discussed in section 3.1 that the salt inhibition started at the 150 mM NaCl treatment (Figure 1), this corresponded to an increase in total potassium concentration which started at this concentration and steadily increased as the salt concentration increased to 200 mM NaCl (Figure 5, Appendix A1).

Environmental stress conditions such as salinity, drought and plant hormones influence the opening, closing of the stomata and the uptake of ions by the plants. It has been reported that external Na^+ negatively impacts intracellular K^+ influx reducing acquisition of this essential nutrient by cells (Hasegawa *et al.,* 2000). Several natural and synthetic cytokinins have been shown to be involved in the stomatal opening. It has also been

reported that application of IAA (Indole Acetic Acid) at low concentrations promote the opening of the stomata (Marten *et al.,* 1991) and to activate $I_{(K+in)}$ providing pathway for the K^+ influx (Blatt and Thiel, 1994; Grabov and Blatt, 1997). As indicated by the results in Figure 5, application of salt to the plants possibly activate the stomatal opening therefore increasing $I_{(K+in)}$ influx into the plants as compared to the salt-kinetin treated plants. It has been shown that application of zeatin or kinetin in *Commelina* decreased the stomatal opening (Blackman and Davies, 1983). According to the results shown above salt-kinetin combination decreased the rate of the stomatal opening/ aperture, this resulted in the decrease of the rate of the inward K^+ channels, decrease in the plasma membrane proton pump providing proton motive force of K^+ uptake, thus leading to the decrease of the total potassium concentration present in the plant. The untreated plants had the highest total potassium concentration; this was expected because the H^+ electrochemical gradient that is responsible for transportation of K^+ ions was not disturbed or altered in any way.

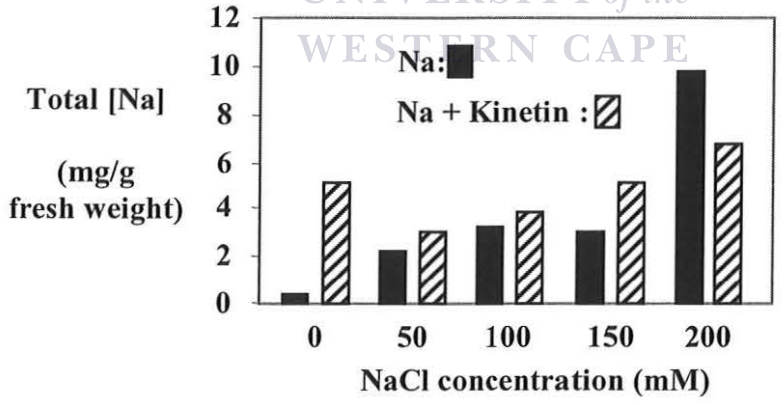


Figure 6: Graph of total sodium [Na] concentration (mg/g fresh weight) versus NaCl concentration (mM) with and without 10 µM kinetin. *A. thaliana* plants were grown for 24 days on various NaCl concentrations

(0, 50, 100, 150, 200) mM (black bars) with and without 10 µM kinetin (striped bars) after which they were harvested, treated and subject to ion analyses via a atomic absorption spectrophotometer.

Similarly, total sodium concentration was also measured in the untreated and treated *A. thaliana* plants. The analysis of the total sodium concentration in the plants showed that the untreated plants had the least total sodium concentration (0.363 mg/g) present (Figure 6, Appendix A2). This was expected since sodium is not one of the essential nutrients required by plants (Mader, 1996). The total sodium concentration in kinetin treated plants (5.051 mg/g) was greater than the 50 mM NaCl (2.165 mg/g), 100 mM NaCl (3.253 mg/g) and 150 mM NaCl (3.097 mg/g) treated plants (Figure 6, Appendix A2). The sodium concentration in kinetin treated plants was also greater than 50 mM NaCl + 10 µM kinetin (3.114 mg/g), 100 mM NaCl + 10 µM kinetin (3.790 mg/g) treated plants. The 150 mM NaCl + 10 µM kinetin (5.09 mg/g) had a similar total sodium concentration to kinetin treated only plants.

The salt treated plants had less total sodium concentration than their counterpart salt-kinetin treated plants at similar (50, 100 and 150) mM NaCl concentrations. There was also a gradual increase in total sodium concentration in both the salt and salt-kinetin treated plants at 50, 100 and 150 mM NaCl concentrations (Figure 6). The plants that had the highest total sodium concentration were those treated with 200 mM NaCl (9.796 mg/g). Plants treated with 200 mM NaCl + 10 µM kinetin (6.67 mg/g) were the most susceptible to salt (Figure 2). The lower total sodium concentration in 200 mM NaCl + 10 µM kinetin treated as compared to the 200 mM NaCl treated plants is possibly related to

the observation that fewer plants germinated on this specific salt-kinetin treatment (20 %) compared to the 200 mM NaCl treatment (40%).

In hypersaline environment after the plants experience salt stress shock the H^+ electrochemical gradient which is involved in the Na^+ and Cl^- ions transport across the plasma membrane is altered (Hasegawa *et al.,* 2000). Influx of Na^+ ions dissipates the membrane potential, thereby facilitating uptake of Cl^- ions down the chemical gradient and also an anion channel has been implicated in this passive flux (Czempinski *et al.,* 1999; Hedrich, 1994; Skerrett and Tyermann, 1994). Physiological data has indicated that Na^+ competes with K^+ for intracellular influx because the cations are transported by common proteins (Blumwald *et al.,* 2000; Niu *et al.,* 1995). It has also been reported that many K^+ transport systems have some affinity to Na^+ (Blumwald *et al.,* 2000; Chrispeels *et al.,* 1999; Schachtman and Liu, 1999). These include inward rectifying K^+ channels, Na^+-K^+ symporter (Schachtman and Schroeder, 1994), K^+ transporters (Fu and Luan, 1998; Santa-Maria *et al.,* 1997; Kim *et al.,* 1998), voltage-dependent, nonselective, outward-rectifying cation channel that mediates Na^+ influx upon plasma depolarisation (Blumwald *et al.,* 2000 and Schachtman *et al.,* 1991).

Our concentration analyses (Figure 5 and Figure 6) for both salt-kinetin and salt treated plants support the observation that the electrochemical gradient is altered. It also appears from our analyses that kinetin increases sodium concentration possibly by increasing the affinity of K^+ transport system for Na^+ ions since salt-kinetin treated plants had less total potassium concentration (Appendix A1).

3.3 Characterization of *Erucastrum strigosum* and *Arabidopsis thaliana* PNP- A sequence

Initially work was done only on *Arabidopsis thaliana* since this plant is the model plant for genetic and molecular studies (Yokoi *et al.,* 2002). However, not sufficient PNP-A protein can be extracted from *A. thaliana* for protein analysis. In order to acquire more plant material for PNP-A protein analysis, *Erucastrum strigosum* a member of the same plant family as *Arabidopsis thaliana* (Brassicaceus) was used for protein extraction.

In *Arabidopsis thaliana*, two irPNP-encoding genes, AtPNP-A and AtPNP-B have been identified and isolated (Ludidi *et al.,* 2002). AtPNP-A encodes a protein of 13998 Da including a predicted signal peptide of 3949 Da and AtPNP-B encodes a protein of 13228 Da including a signal peptide 3201 Da (Ludidi *et al.,* 2002). AtPNP-A protein has 37% identity to the AtPNP-B protein, the latter protein has been annoted as a blight associated protein homologue since it showed a high degree (54 %) of identity to the CjBAp12 protein. CjBap12 is a blight induced protein of unknown function isolated from *Citrus jambhiri* (Ceccardi *et al.,* 1998) and shows significant homology to expansins (Cosgrove, 1999).

Expansins are extracellular proteins which are involved in plant cell wall loosening but they lack hydrolytic activity (McQueen-Mason *et al.,* 1992; McQueen-Mason and Cosgroove, 1995). Expansins are functionally defined to induce long-term pH-dependent

extension and enhance stress relaxation of plant cell walls (McQueen-Mason *et al.,* 1992; McQueen-Mason and Cosgrove, 1995).

In order to verify that the *Erucastrum strigosum* genome also contained a PNP-A gene, a recombinant plasmid containing the *Erucastrum* PNP-A gene (pCREsPNPA) which was previously partially sequenced by M. Maqungo (personal communication) was re-sequenced (Figure 7) in order to determine the nucleotide similarities between the *A. thaliana* PNP-A gene and the *Erucastrum* PNP-A gene. The *A. thaliana* PNP-A gene (AC00572) was previously sequenced (Ludidi *et al.,* 2002).

```
ATGGCAGTAAAATTTGTGGTAGTGATGATAGTGTTTGCACAAATCTTAGC
TCCAATCGCTGAGGCTGCTCAAGGAAAAGCTGTCTATTACGACCCTCCCT
ACACTAGGTCTGCGTGTTACGGAACACAACGTGAGACGCTGGTAGTCGGA
GTCAAGAACAATTTGTGGCAAAATGGTCGAGCTTGTGGTCGGAGGTACAG
GGTGCGATGCATTGGTGCTACATACAACTTTGACAGAGCAGCACCGGGCG
GTACCGTAGACGTGAAGGTAGTTGATTTCTGCCGGGAGCCTTGCAACGGT
GACCTTAATCTCTCTCGTGACGCTTTTCGGGTTGTCGCTAATACTGATGC
CGGTAACATTCGCGTCGTATACACACCGTAA
```


Figure 7: Nucleotide sequence of the *E. strigosum* PNP-A gene. Plasmid pCREsPNPA was sequenced using the ABI Prism™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) at the University of Stellenbosch Sequencing Department.

The sequence obtained was analysed and translated into the corresponding protein sequence using the DNAMAN ver. 4.13 (LYNNON BIOSOFT, Canada) software package (Figure 8).

```
  1 MAVKFVVVMI VFAQILAPIA EAAQGKAVYY DPPYTRSACY GTQRETLVVG VKNNLWQNGR
 61 ACGRRYRVRC IGATYNFDRA CTGRTVDVKV VDFCREPCNG DLNLSRDAFR VVANTDAGNI
121 RVVYTP
```

Figure 8: Protein sequence of the *E. strigosum* PNP-A gene translated from the nucleotide sequence using the DNAMAN ver. 4.13 (LYNNON BIOSOFT, Canada) software package.

The sequence results revealed that the *E. strigosum* PNP-A gene was 381 bp in length and sequence analyses indicated that the *E. strigosum* PNP-A sequence had high nucleotide homology to the *A. thaliana* PNP-A gene sequence with a 98.95 % sequence identity at the DNA level. At the protein level the amino acid identity was 99.21 % between the *A. thaliana* PNP-A and the PNP-A from *E. strigosum*. There was only a single amino acid change between the *A. thaliana* and *E. strigosum* PNP-A protein sequences at amino acid position 111 where the isoleucine in the *A. thaliana* PNP-A protein was changed to a valine in the corresponding *E. strigosum* PNP-A sequence. Both these amino acids belong to the aliphatic group and therefore the amino acid change between the two PNP-A molecules was a conservative change. The predicted molecular weight of the protein is 14 kDa and predicted length is 126 amino acids.

This result indicates that the PNP-A gene occurs on the *E. strigosum* genome and the near identical conservation of amino acids at the protein level is significant at it indicates that the *E. strigosum* PNP-A protein shares a similar if not identical function as the *A. thaliana* PNP-A protein, as is also most likely involved in water and ion homeostasis.

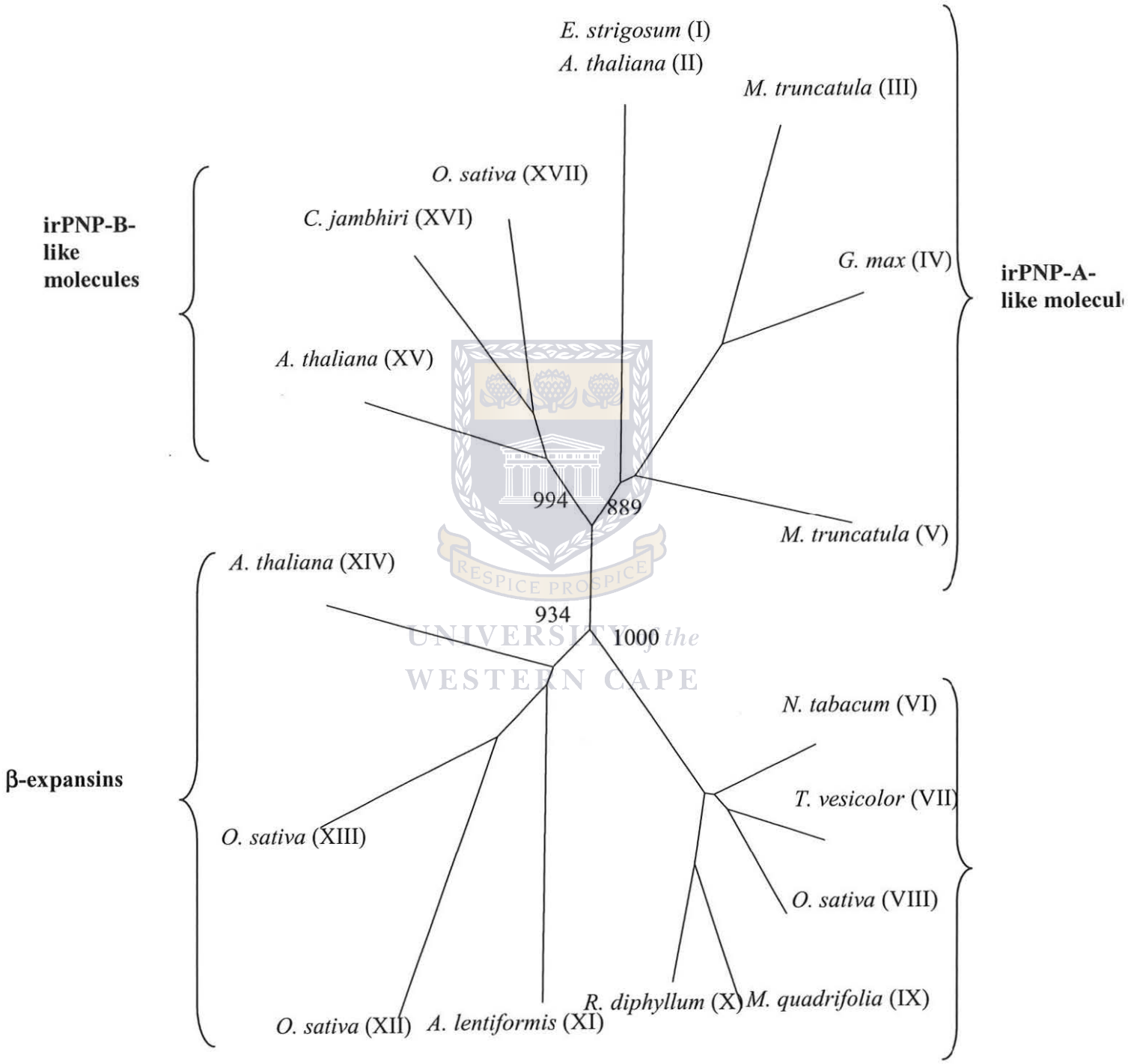


Figure 9: Phylogenetic tree representing the evolutionary relationship between irPNP-like molecules to α–expansins and β-expansins. Branch lengths are approximately proportional to the amount of sequence difference. The scale of 0.1 indicates 10% amino acid sequence divergence. Bootstrap values were taken from 1000 replicates. The accession numbers for the protein sequences were obtained from the TIGR (http://www.tigr.org/tdb/tgi.shtml) and Genbank (http://www.ncbi.nlm.nih.gov/Entrez/) databases. Sequence identification: **[I]** AY093582 **[II]** AAD08935 **[III]** TC29780 **[IV]** TC64839 **[V]** BE943215 **[VI]** AAC96081 **[VII]** T50660 **[VIII]** AAF62180 **[IX]** AAF17570 **[X]** AAF17571 **[XI]** BAB20817 **[XII]** AAB61710 **[XIII]** AAF72983 **[XIV]** At2g20750- chromosomal locus **[XV]** CAB79756 **[XVI]** AAD03398 **[XVII]** OSM11811

Phylogenetic analyses of the PNP-A molecules and expansins revealed that both the *A. thaliana* and *E. strigosum* PNP-A molecules belonged to the irPNP-A-like family (Figure 9). These sequence and phylogenetic analyses allowed us to prepare for protein analyses of the PNP-A protein extracted from *Erucastrum strigosum*.

3.4 Preliminary analysis of PNP-A protein extracted from untreated *Erucastrum strigosum* plant leaves

Maintenance of water and solute homeostasis is a key requirement for all living systems. In animals the natriuretic peptides regulates water and salt balance in the heart, kidney, vascular tissue and adrenal gland (Koller and Goeddel, 1992). In plants similar peptides, called irPNP, immunoreactant plant natriuretic peptide, has been isolated and identified (Billington *et al.,* 1992). The immunoreactant plant natriuretic peptides (irPNP) have been shown to be involved in several ion transport systems of potassium (K^+), calcium (Ca^{+2}), sodium (Na^+), chloride (Cl^-) and water transport in plants (Pharmawati *et al.,* 1999). It also been shown that irPNP promote stomatal opening (Billington *et al.,* 1997).

It has been reported that AtPNP-A is possibly expressed at the transcriptional level in unstressed plant leaves as the PNP-A gene was obtained via RT-PCR (Ludidi *et al.*, 2002). Our sequence analysis also indicated that a similar PNP-A protein molecule as in *A. thaliana* occurs in *E. strigosum* and therefore we sought to isolate the PNP-A protein from *E. strigosum* plant leaves.

E. strigosum plants that were grown for 35 days and not subject to any kind of treatment were obtained from G. Ngxaba (personal communication). Protein extraction was performed on the plant leaves as discussed in section 2.5. The crude protein extract was run on the SDS-PAGE and silver stain was used for detection as discussed in section 2.9.

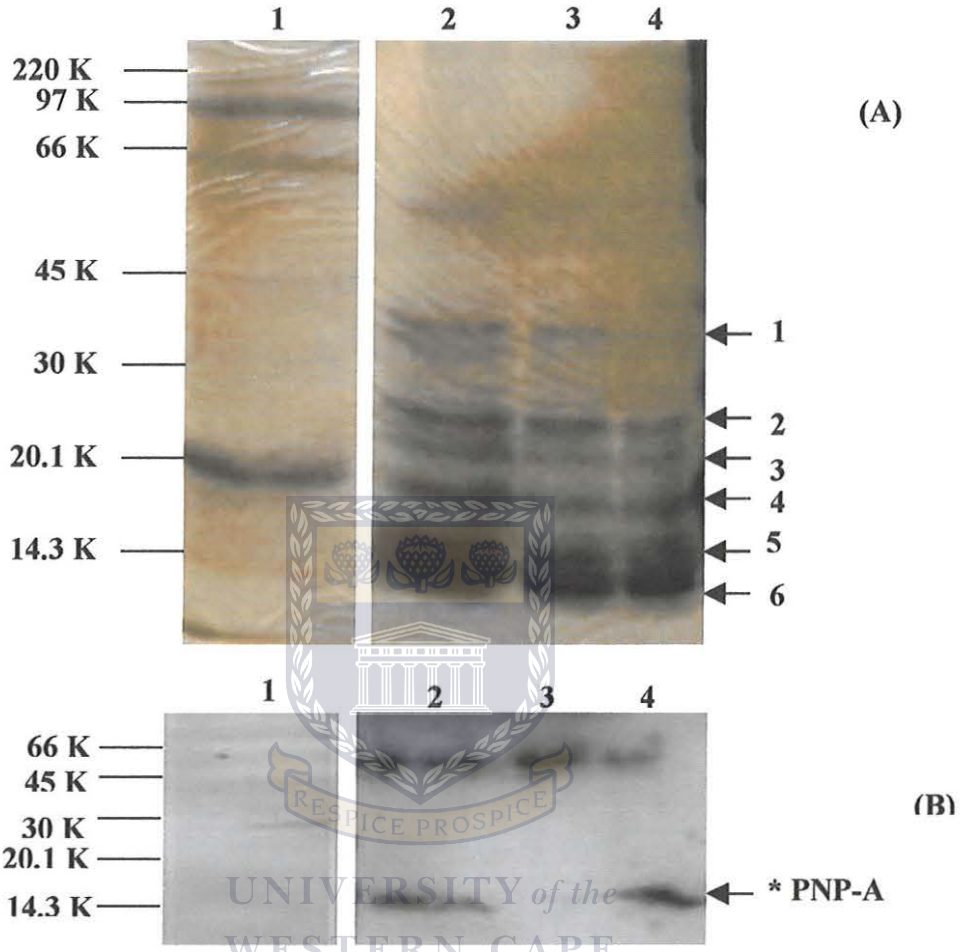


Figure 10: SDS-PAGE and western blot analysis of protein extracted from untreated *E. strigosum* plant leaves. (A): SDS-PAGE analysis of protein extracted from untreated *E. strigosum*. Lane 1: Rainbow Marker 756 (Amersham, UK); Lane 2: Untreated E.S; Lane 3: Untreated E.S; Lane 4: Untreated E.S and (B) Western blot analysis of protein extracted from untreated *E. strigosum* plant leaves. The irPNP was detected using rabbit ant-ANP (1-28 human) antibody and detected using the ECL Plus Western Blotting detection system (Amersham, UK). Lane 1: Rainbow Marker 756; Lane 2: Untreated E.S; Lane 3: Untreated E.S; Lane 4: Untreated E.S. Arrow (asterix) indicates the position of the PNP-A protein after detection.

The SDS-PAGE analysis (Figure 10A) revealed that crude protein extracted from untreated *E. strigosum* plant leaves produced five bands with different molecular weights. The molecular weight of bands ranged from ~40 kDa to ~14 kDa. The size of the PNP-A protein falls within the 14 kDa range (section 3.3).

Western blot analysis of the protein extracted from untreated *E. strigosum* using anti-ANP antibody revealed that PNP-A was present in the crude protein extracted from *E. strigosum* plant leaves with molecular weight of ~14 kDa (Figure 10B). This result together with the sequence analysis confirmed that PNP-A is present in *E. strigosum* plant leaves and the protein's molecular weight (~14 kDa) is the same as predicted from sequence analysis.

3.5 Preliminary analysis of PNP-A protein extracted from *E. strigosum* plant leaves from plants treated with salt-kinetin

Droughts, salinity, freezing are stresses that cause adverse effects on the growth of plants and the productivity of crops. The physiological response to these stresses arises out of changes in cellular gene expression and expression of a number of these genes have been shown to be induced by these stresses (Shinozaki and Yamaguchi-Shinozaki, 1996; Thomashow *et al.,* 1994). The products of these genes can be classified into two groups: those that directly protect against environmental stresses and those that regulate gene expression and signal transduction in stress response (Shinozaki *et al.,* 1997). Previously it has been demonstrated that irPNP are involved in the stomatal guard cells (Gehring, 1999; Pharmawati *et al.,* 2001), ion transport (Pharmawati *et al.,* 1999; Maryani *et al.,*

2000) and osmoticum-dependent volume regulation (Maryani *et al.*, 2001). Similarities in biological responses to ANP and irPNP in plants (Gehring *et al.*, 1999) indicates that irPNP-like (AtPNP-A and AtPNP-B) molecules might have a role in homeostasis much like ANPs have in vertebrates.

We therefore sought to understand whether the PNP-A was expressed in *E. strigosum* plants treated with salt and kinetin and whether the treatment affected the PNP-A expression using SDS-PAGE and western blot analysis (Figure 11A and 11B).

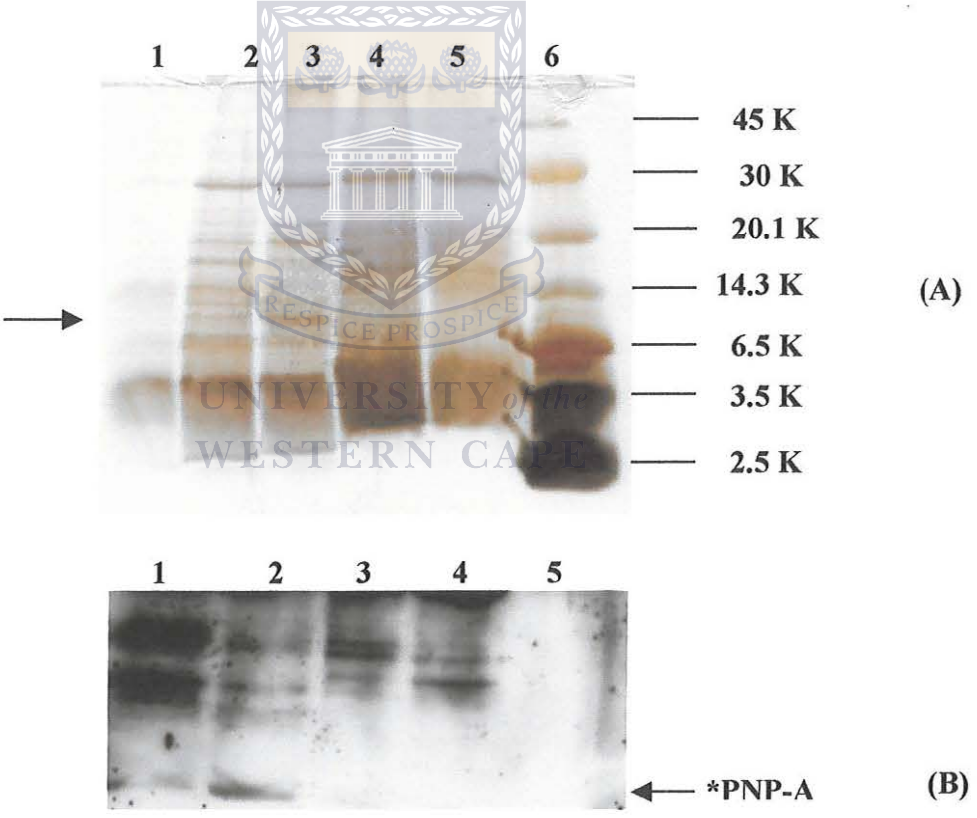


Figure 11: SDS-PAGE and western blot analysis of protein extracted from *E. strigosum* plant leaves. The plants were treated with different salt concentrations (0, 100, 200, 300) mM NaCl + 4 µM kinetin. (A): SDS-PAGE analysis of protein extracted from treated *E. strigosum*. (A) Lane 1: E.S 4 µM K + 300 mM

NaCl; Lane 2: E.S 4 µM K + 200 mM NaCl; Lane 3: E.S 4 µM K + 100 mM NaCl; Lane 4: E.S 4 µM K + 0 mM NaCl; Lane 5: Untreated E.S; Lane 6: Rainbow marker 755 (Amersham, UK). (B) Western blot analysis of protein extracted from treated *E. strigosum* plant leaves. The irPNP was detected using rabbit ant-ANP (1-28 human) antibody and detected using the ECL Plus Western Blotting detection system (Amersham, UK). (B) Lane 1: Untreated E.S; Lane 2: E.S 4 µM K + 0 mM NaCl; Lane 3: E.S 4 µM K + 100 mM NaCl; Lane 4: E.S 4 µM K + 200 mM NaCl; Lane 5: E.S 4 µM K + 300 mM NaCl. Arrow (asterix) indicates the position of the PNP-A protein after detection.

Protein extractions were performed on *Erucastrum strigosum* plants leaves. The plants obtained from G. Ngxaba (personal communication) were grown for 35 days, treated with 4 µM kinetin and different NaCl (0, 100, 200, 300) mM concentrations. Subsequently, SDS-PAGE and western blot analysis was performed on the extracted protein.

It was observed that a protein band of ~14 kDa was present in *E. strigosum* plant leaves extracted from the untreated plants and in all the plants treated with NaCl + 4 µM kinetin (Figure 11B). However, the 14 kDa band were very faint in the 300 mM + 4 µM kinetin treatment possibly due to low protein concentration.

Western blot analysis of the protein extracted from untreated and treated *E. strigosum* plant leaves using irPNP antibody revealed that PNP-A was present in the untreated plants and in the plants treated only with 4 µM kinetin and not present in the other plants treated with both salt and kinetin. The molecular weight of the band detected was ~14 kDa (Figure 11B). The reason no bands were obtained in the NaCl (100, 200 and 300) mM + 4 µM kinetin treatments could have been due to loading of insufficient protein or incomplete transfer during the western blot procedure.

It was therefore decided to determine the regulation of the *E. strigosum* PNP-A protein expression using BIOCAD affinity purification and subsequent western blot analysis.

3.6 Analysis of regulation of PNP-A expression extracted from untreated and treated *E. strigosum* plant leaves

IrPNPs were initially isolated through the use of immuno-affinity purification via an antibody directed against ANP, vertebrate signal peptide, which has a role in water and solute homeostasis (Billington *et al.,* 1997; Gehring, 1999). Natriuretic peptides, ANPs and irPNPs have been shown to affect ion transport (Pharmawati *et al.,* 1999; Maryani *et al.,* 2000; Pharmawati *et al.,* 2001) across the plant cell membranes. Furthermore, water transport has been shown to be affected by ANP in vertebrate systems (Wolfensberger *et al.,* 1994; Han *et al.,* 1998) and both ANP and irPNP modulate osmoticum dependent water transport (Maryani *et al.,* 2001) in protoplast, an experimental system without cell walls.

To understand the molecular nature of the irPNP-like molecule PNP-A, irPNPs were isolated and partially sequenced from potato (Maryani *et al.,* 2001) and at the genomic level, the *A. thaliana*, PNP-A gene encoding and irPNP-A was identified and isolated (Ludidi *et al.,* 2002). It was found, that a section of the predicted *A. thaliana* PNP-A protein sequence had homology to the vertebrate ANP epitope thus the irPNP antibody should recognize the A. thaliana PNP-A protein (Ludidi *et al.,* 2002). This also indicates, that similar to irPNPs, PNP-A is involved in water and ion homeostasis. Therefore to

further understand the involvement of PNP-A in ion homeostasis in plants and the effect that salt has on PNP-A, protein was extracted from salt treated *E. strigosum* plant leaves and was run on the BIOCAD sprint system.

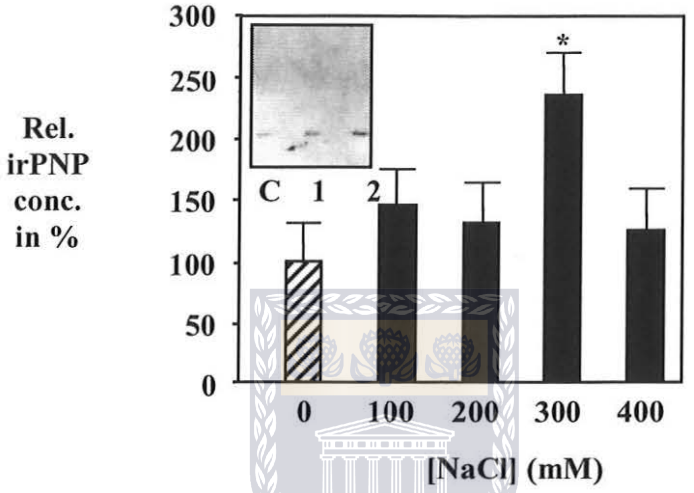


Figure 12: BIOCAD immuno-affinity purification of PNP-A protein extracted from *E.strigosum* plant leaves. The plants were treated with different NaCl concentrations (0 (control), 100, 200, 300 and 400) mM for 35 days. PNP-A protein was then extracted and run on the BIOCAD using the irPNP-A affinity column. The percentage (%) PNP-A is expressed relative to the control. The control is the amount of eluted PNP-A protein versus the total protein and is expressed as 100%. Inset: Western blot analysis: Lane 1: control (0 mM NaCl); Lane 2: 100 mM NaCl; Lane 3: 300 mM NaCl.

Protein extractions were performed on *Erucastrum strigosum* plant leaves grown for 35 days treated with different NaCl (0,100,200,300,400) mM obtained from G.Ngxaba (personal communication) and the extracts were affinity purified as discussed in section 2.6. The ratio for the sample with no salt treatment was designated the value of 100%. As indicated above in Figure 12 the BIOCAD Sprint System analyses revealed that the PNP-

A level is highest in the sample treated with 300 mM NaCl as compared to the control (0), 100, 200 and 400 mm NaCl treated samples. Using the control as a reference this means that there was 150% increment of PNP-A at 300 mM NaCl. At 100 mM NaCl there was 50% increment of PNP-A level but as the NaCl was increased to 200 mM there was a decrease of approximately 20% comparing the plants treated with 100 and 200 mM NaCl. If we compare the control and the 200 mM NaCl treated samples there was 30 % increase of PNP-A at 200 mM NaCl. Comparing 200 mM and 400 mM NaCl treated samples there was no difference in PNP-A levels. The PNP-A level was still at 30 % increment with reference to the control. The graph in Figure 12 indicates that PNP-A is involved in ion homeostasis in the plant and the PNP-A levels are affected by the NaCl concentrations. The PNP-A was up-regulated at 300 mM NaCl.

It has been previously reported that irPNPs are involved in the ion transport in plants (Pharmawati *et al.,* 1999; Maryani *et al.,* 2000; Pharmawati *et al.,* 2001). It has also been shown that the *Arabidopsis thaliana* AtPNP-A and ANP share some sequence homology (Ludidi *et al.,* 2002) and this may implies that irPNPs are involved in ion homeostasis of the plant.

To further determine that this molecules is PNP-A, western blot analysis was performed as discussed in section 2.11. As shown in the inset of Figure 12 western blot analyses revealed that there were single bands with approximately 14 kDa in the control (0), 100 and 300 MM NaCl treated samples, which implies that this is a PNP-A molecule. The

band intensity at 300 mM NaCl was substantially increased compared to the control (Figure 12)

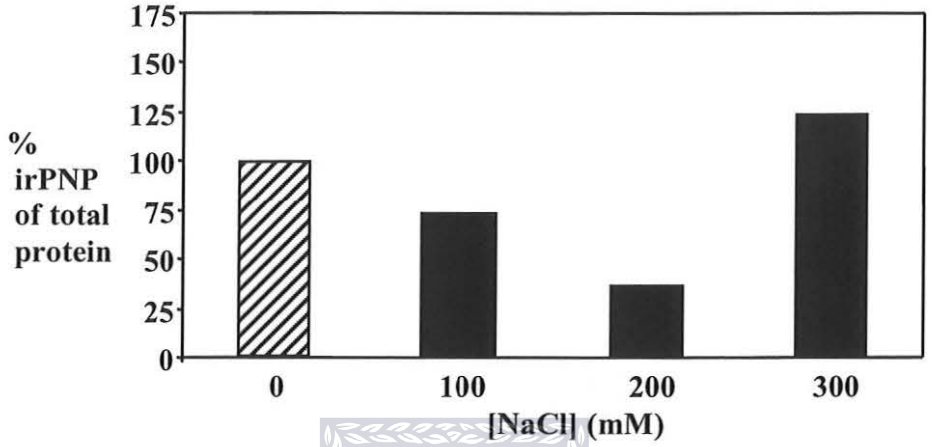


Figure 13: BIOCAD immuno-affinity purification of PNP-A protein extracted from *E.strigosum* plant leaves. The plants were treated with different NaCl concentrations (0 (control), 100, 200, and 300) mM and 4 µM kinetin for 35 days. PNP-A protein was then extracted and run on the BIOCAD using the irPNP-A affinity column. The percentage (%) PNP-A is expressed relative to the control. The control is the amount of eluted PNP-A protein versus the total protein and is expressed as 100%.

To further characterize PNP-A involvement in ion homeostasis and its effect by salt-kinetin combination treatment, protein extraction and affinity purification was performed also on *Erucastrum strigosum* plants leaves grown for 35 days treated with both 4 µM kinetin and different NaCl (0, 100, 200, 300) mM concentrations obtained from G.Ngxaba (personal communication) as discussed in 2.5 and 2.6 sections respectively. The ratio for the sample with no NaCl added was designated the value 100%. As shown in Figure 13 *Erucastrum strigosum* treated with both 4 µM kinetin and 300 mM NaCl has the highest PNP-A level as compared to 4 µM kinetin and NaCl (0, 100, 200) mM treated samples. The PNP-A level has a steady decrease from 0 to 200 mM NaCl but greatly

increases at 300 mM NaCl. Using the 0 mM NaCl + 4 μM kinetin bar in the graph as a reference, there is a 25% decrease in PNP-A level at 100 mM NaCl + 4 μM kinetin and there is a further 50% decrease in PNP-A level at 200 mM NaCl + 4 μM kinetin. As the salt concentration was increased to 300 mM NaCl + 4 μM kinetin there was a 25% increase in the PNP-A levels.

Comparing Figure 12 and Figure 13, the salt treated plants have more PNP-A than the salt-kinetin treated samples. With salt treated plants there is an increment at all different salt concentrations (100, 200, 300, 400) mM but with salt-kinetin treatment there is a decrease of PNP-A at 100, 200 mM NaCl and only an increment at 300 mM NaCl. In both Figure 12 and Figure 13 salt-treated and salt-kinetin treated samples respectively, PNP-A level is at its highest at 300 mM NaCl treatment. As previously discussed in Figure 1, 2, and 3 that the *Arabidopsis thaliana* plants treated with both kinetin and salt were more susceptible to salt than the salt treated plants. It has also been shown that *Erucastrum strigosum* and *Arabidopsis thaliana* sequence analyses indicated that the *E. strigosum* PNP-A sequence had high nucleotide homology to the *A. thaliana* PNP-A gene sequence with a 98.95 % sequence identity at the DNA level. At the protein level the amino acid identity was 99.21 % sequence between the *A. thaliana* PNP-A and the PNP-A from *E. strigosum*. This confirms that *E.strigosum* and *A. thaliana* are very closely related. Therefore it was expected that PNP-A levels in *Erucastrum strigosum* plants treated with salt-kinetin will be less than plants treated only with salt because the ion transport/ electrochemical gradient/ biochemical steady state that is important for the growth of the plants was more altered than in the salt-treated plants. Since PNP-A is

involved in water and ion movement the results in Figure 12 and Figure 13 shows that PNP-A activity is high at 300mM NaCl and it is affected by the presence of either salt or salt-kinetin treatment.

3.7 Characterisation of PNP-A using BIOCAD Sprint system, MALDI TOF and silver stained SDS PAGE gels

Plants could contain more than one type of natriuretic peptide, since the eluted fraction of immunoreactant plant natriuretic peptide (irPNP) were shown to consist of several molecules with different molecular weights ranging from 3kDa to 16kDa (Pharmawati *et al.,* 1998b). The irPNP C and the N-termi have been partially sequenced from the potato *Solum tuberosum* and the SDS gel of irPNP showed major and minor lower band, indicating that two irPNPs were isolated (irPNP-A and irPNP-B) (Maryani *et al.,* 2001). Both peptides showed a significant degree of similarity in the N-terminal region and in addition, the two peptides showed high degree of comparability with CjBAp12, a blight induced protein of unknown function from citrus jambiri (Ceccardi *et al.,* 1998). Also an *Arabidopsis thaliana* transcript (AtPNP-A) encoding an immunoreactant plant natriuretic peptide (irPNP) analogue was identified and isolated (Ludidi *et al.,* 2002). The encoded protein also showed similarity with CjBAp12 (Ludidi *et al.,* 2002). The genomic sequence of AtPNP-A is 478 bp long and contains one predicted 100 bp long intron thus the protein consist of 126 amino acids (approximately 14 kDa) (Ludidi *et al.,* 2002). In addition AtPNP-A encodes a protein of 13 998 Da including a predicted signal peptide of 3 949 Da and AtPNP-B encodes a protein of 13 228 Da including a predicted signal peptide of 3 201 Da (Ludidi *et al.,* 2002).

To characterize the *E. strigosum* PNP-A protein and purify the protein, the protein was extracted from untreated *Erucastrum strigosum* plant leaves and then affinity purified using Anti-ANP column in the BIOCAD Sprint system in order to isolate *Erucastrum strigosum* PNP-A.

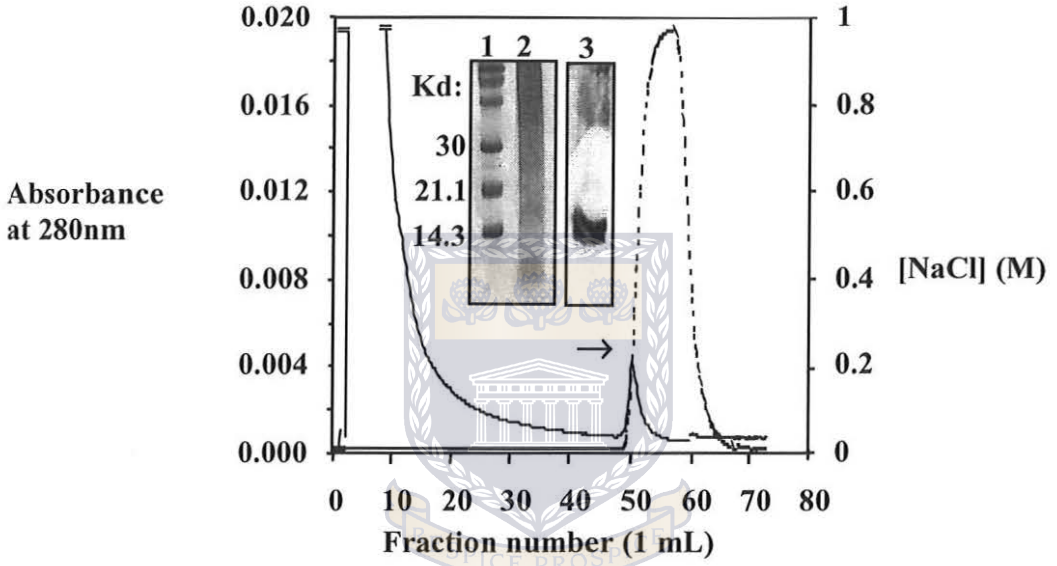


Figure 14: BIOCAD Immunoaffinity purification of untreated *Erucastrum strigosum* PNP-A protein extract from leaves and shoot tissue. The arrow (→) indicates the release of bound immuno-reactant protein with increasing ionic strength. The solid line is the protein trace and the broken line represents the NaCl concentration. Inset: Silver stained SDS PAGE gel Lane:1 Rainbow Marker 755; Lane: 2 Unbound eluted protein fractions; Lane 3: EsPNP-A protein

The elution profile (Figure 14) shows that when NaCl was not applied to the column large amounts of PNP-A protein was not bound to the column as indicated by the first peak > 95% and also indicated by the silver stained SDS PAGE gel in Lane 2 (inset). The baseline was reached at fraction 40. When NaCl was applied to the column with subsequent increase in concentration from 200 mM to 1 M the bound PNP-A protein

fractions were released from the affinity column indicates by the arrow (Figure 14). The PNP-A protein was released by 200 mM NaCl application and the salt concentration was increased to remove any PNP-A proteins that might be left in the column.

Silver stained SDS PAGE gel analyses (Figure 14) was used for the detection of PNP-A protein. The SDS PAGE analyses in Lane 3 (inset) revealed that PNP-A protein had two bands with molecular weights of ~ 13 kDa and ~10 kDa. It has been previously shown that plants could have more than one type of natriuretic peptide, since the eluted fraction of immunoreactant plant natriuretic peptide (irPNP) were shown to consist of several molecules with different molecular weight ranging from 3 kDa to 16 kDa (Pharmawati *et al.,* 1998b). The ~13 kDa protein band could represent the PNP-A protein with the signal peptide (13 998 Da) and the ~10 kDa protein band could represent the PNP-A without the signal peptide (3 949 Da) (Ludidi *et al.,* 2002). This indicates that PNP-A existed in two forms in untreated *E. strigosum* plants.

Following these findings we sought to characterize PNP-A in *E. strigosum* in response to salt stress with and without kinetin by using the SDS PAGE gel, MALDI TOF and the BIOCAD Sprint system.

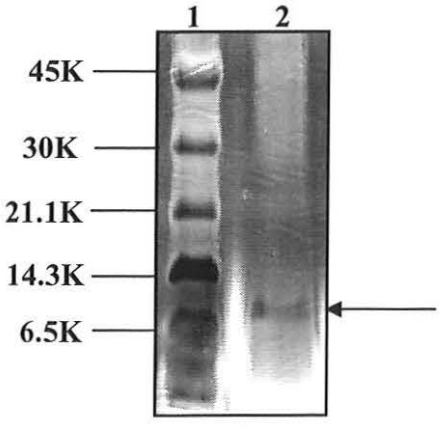


Figure 15: SDS-PAGE analysis of eluted *E. strigosum* PNP-A protein fractions obtained from the anti-ANP affinity column. *E.strigosum* (E.S) plants were treated with different NaCl (0, 100, 200,300) mM concentrations and 4 μM kinetin (K). The SDS-PAGE was stained with silver stain (). Lane 1: Rainbow Marker 755 (Amersham, UK; Lane 2: E.S PNP-A eluted and pooled protein fraction NaCl (0, 100, 200, 300) mM + 4 μM K. The arrow indicates a double band with a calculated molecular weight of ~8 Da and 9 Da respectively .

The protein extractions of *Erucastrum strigosum* plant leaves treated with 4 μM kinetin and different NaCl (0, 100, 200, 300) mM concentrations were affinity purified using BIOCAD Sprint system as discussed in 2.6 section. The resulting fractions were combined and freeze dried (see section 2.5) and the PNP-A protein was visualised with silver stain (Figure 15).

SDS-PAGE analysis as shown in the above figure revealed the presence of two protein bands having a molecular mass of ~8 and 9 Da respectively. These protein were eluted from the anti-ANP column thus these two bands must be processed forms of the PNP-A protein. To determine the exact size of the two PNP-A protein bands the sample was further analysed using MALDI TOF (Figure 16).

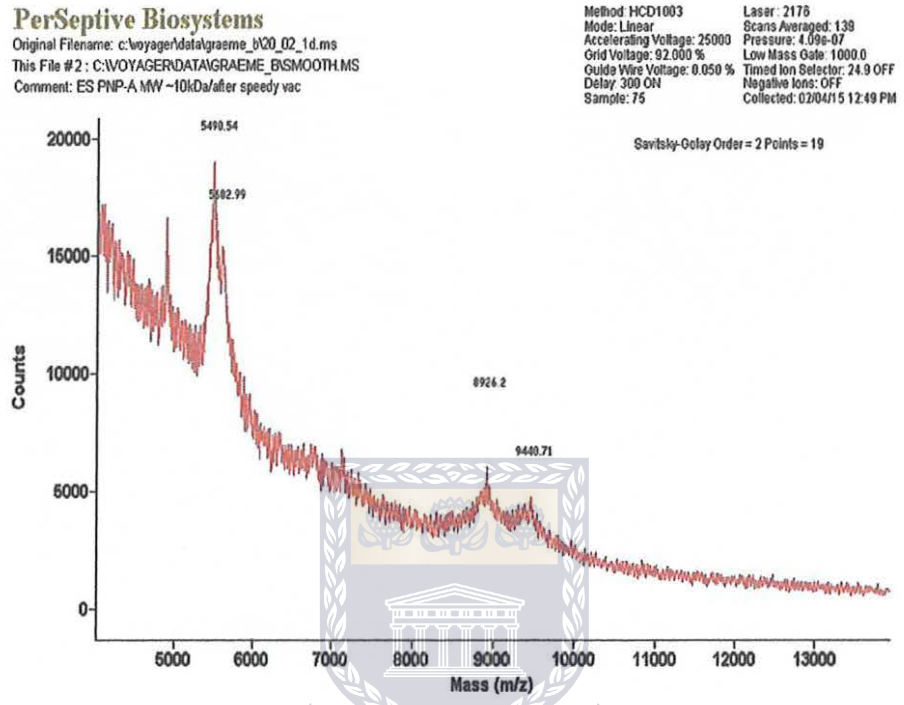


Figure 16: MALDI TOF mass spectrum analysis of *E. strigosum* PNP-A protein eluted from the anti-ANP column. The *E. strigosum* plants were treated with different NaCl concentrations (0,100, 200, 300) mM and 4 µM kinetin. The extracted proteins were run on the Anti-ANP affinity of column, PNP-A eluted, the samples pooled and then sent for MALDI-TOF analysis at the University of Cape Town, S. Africa.

This revealed that the two bands had a molecular weight of 8926.2 Da and 9440.71 Da. This cannot be the PNP-A protein without the signal peptide because when the molecular weight of both protein products are combined it is greater than the PNP-A protein with the signal peptide (*A. thaliana* PNP-A protein: 13 998 Da). This must therefore represent processed forms of the PNP-A protein. This processing might occur as a response to salt stress.

3.8 Northern and slot blot analysis of PNP-A in salt treated *Erucastrum strigosum*

It was observed with the BIOCAD immuno-affinity purification analyses of PNP-A protein extract from *Erucastrum strigosum* that PNP-A was up-regulated at 300 mM NaCl. In order to see whether the up-regulation of PNP-A protein observed with BIOCAD immuno-affinity purification analysis was reflected at the transcriptional level, northern and slot blots were done.

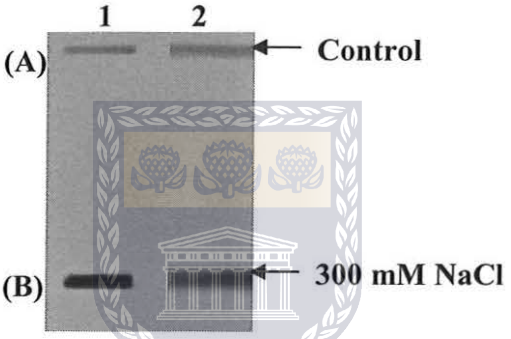


Figure 17: PNP-A slot blot of salt treated *Erucastrum strigosum*. Lane 1A: Untreated *E.strigosum* RNA; Lane 1B 300mM NaCl treated *E.strigosum* RNA; Lane 2A : Untreated *E.strigosum* RNA; Lane 2B: 300 mM NaCl treated *E.strigosum* RNA. Lane 1 was probed with a DIG labelled *Arabidopsis thaliana* PNP-A RNA probe and Lane 2 was probed with a DIG labelled Actin RNA probe

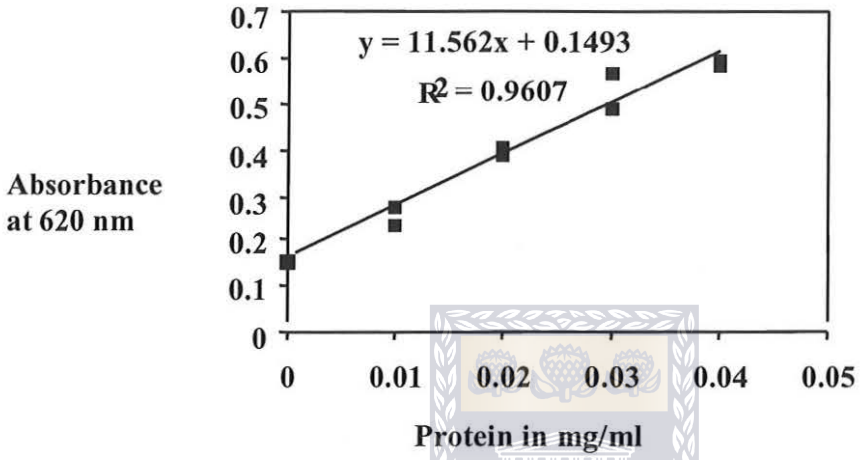
To see the effect of salt on *E.strigosum* PNP-A on transcriptional level, northern and slot blot analysis was performed as discussed in 2.14 and 2.15 section. The northern blot did not produce a signal because of the probe used and detection method followed was not sensitive enough but the RNA extraction used was not degraded (Figure A2, Appendix). The AtPNP-A probe was prepared again and we opted to do the slot blot (Figure 17). The probes synthesis, labelling and slot blot was followed as discussed in section 2.14 and

2.15. Actin was used as a control because the level of Actin mRNA remains constant in eukaryotic cells irrespective of stress applied.

RNA slot blot analysis revealed that there was a 1.5 fold difference in signal intensity between the RNA obtained from untreated *E. strigosum* probed with the *A. thaliana* PNP-A probe (Figure 17 1A) and the Actin probe (Figure 17 1B). There was a 0.5 fold difference in signal intensity between the RNA obtained from *E. strigosum* treated with 300 mM NaCl. Comparison of the control with the 300 mM NaCl treatment showed that there was a three-fold difference between the control and treated plants. This indicated that there was more PNP-A mRNA transcript under NaCl stress compared to the control. This correlates well with the data obtained from BIOCAD analysis where the PNP-A protein was also up-regulated at 300 mM NaCl stress.

4. Appendix A

Figure A1. Standard graph of *Erucastrum strigosum* protein concentration determination



PNP-A northern blot analysis of *Erucastrum strigosum* treated with salt and salt-kinetin

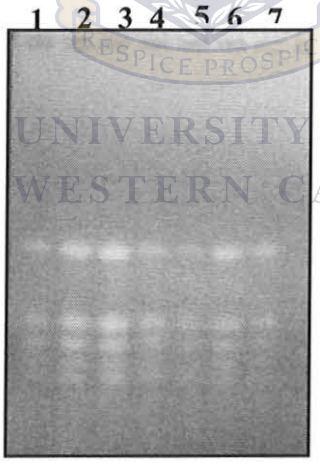


Figure A2: Purified RNA extractions from treated (salt, salt-kinetin) and untreated *Erucastrum strigosum* separated by agarose-formaldehyde electrophoresis, probed with *Arabidopsis thaliana* PNP DiG-labelled . Lane 1: untreated E.S; Lane 2: E.S 4 µM K + 0 mM NaCl; Lane 3: E.S 0 K + 300 mM NaCl; Lane 4: E.S 300 mM NaCl + 4 µM K; Lane 5: E.S 300 mM NaCl + 4 µM K; Lane 6: E.S 0K + 400 mM NaCl; Lane 7: E.S 400 mM NaCl + 4 µM K

Table A1: Concentration of total potassium (K) element fresh weight (mg/g)

0 mM NaCl	5.514
10 µM kinetin	2.664
50 mM NaCl	2.871
50 mM NaCl + 10 µM kinetin	1.397
100 mM NaCl	2.0001
100 mM NaCl + 10 µM kinetin	1.176
150 mM NaCl	2.707
150 mM NaCl + 10 µM kinetin	1.99
200 mM NaCl	2.782
200 mM NaCl + 10 µM kinetin	2.37

Table A2: Concentration of total sodium (Na) element fresh weight (mg/g)

0 mM NaCl	0.363
10 µM kinetin	5.051
50 mM NaCl	2.165
50 mM NaCl + 10 µM kinetin	3.114
100 mM NaCl	3.253
100 mM NaCl + 10 µM kinetin	3.790
150 mM NaCl	3.097
150 mM NaCl + 10 µM kinetin	5.09
200 mM NaCl	9.796
200 mM NaCl + 10 µM kinetin	6.67

5. Summary and outlook

The data presented suggest that the application of kinetin and salt in *Arabidopsis thaliana* enhances the plant's susceptibility to salt thereby accelerating the plant's senescence which leads to early plant death. The data has also shown that irPNP-like molecules occur in *Erucastrum strigosum* and this plant natriuretic peptide (EsPNP-A) is similar to the *Arabidopsis thaliana* plant natriuretic peptide (AtPNP-A) at the DNA and protein level. This indicated that both molecules might have similar functions as irPNPs, which are involved in ion homeostasis.

The role of EsPNP-A in ion homeostasis was indicated by the effect of kinetin and salt stress on *E. strigosum* treated plants where EsPNP-A was up-regulated in response to salt stress. This suggested that the irPNP-like molecule, EsPNP-A, might have a function in the abiotic stress response.

Future experiments could again determine whether the up-regulation of the EsPNP-A observed at the protein level is reflected at the transcriptional level using Northern hybridisation analysis. Production of recombinant PNP-A protein would also assist in elucidation of the structure of the PNP-A molecule and how addition of this molecule affects the plants response to salt stress.

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